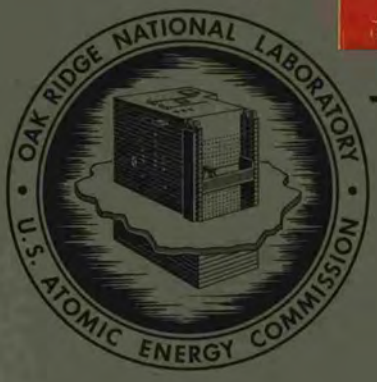
ORNL-4412
Progress

BIOLOGY DIVISION
ANNUAL PROGRESS REPORT
FOR PERIOD ENDING DECEMBER 31, 1968



OAK RIDGE NATIONAL LABORATORY
operated by
UNION CARBIDE CORPORATION
for the
U.S. ATOMIC ENERGY COMMISSION

ORNL-4412
Progress

Contract No. W-7405-eng-26

BIOLOGY DIVISION

ANNUAL PROGRESS REPORT

For Period Ending December 31, 1968

R. F. Kimball, Director
S. F. Carson, Deputy Director

ISSUED

JULY 1969

OAK RIDGE NATIONAL LABORATORY
Oak Ridge, Tennessee
operated by
UNION CARBIDE CORPORATION
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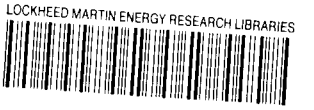

Reports previously issued in this series are as follows:

ORNL-13	Period Ending February 29, 1948
ORNL-61	Period Ending May 31, 1948
ORNL-150	Period Ending August 31, 1948
ORNL-220	Period Ending November 30, 1948
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ORNL-1026	Period Ending May 10, 1951
ORNL-989	Period Ending August 10, 1951
ORNL-1167	Period Ending November 10, 1951
ORNL-1244	Period Ending February 10, 1952
ORNL-1297	Period Ending May 10, 1952
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ORNL-1456	Period Ending November 10, 1952
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ORNL-2997	Period Ending August 15, 1960
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Publications and Lectures

PUBLICATIONS

During the period January 1 through December 31, 1968, 579 manuscripts written or edited by members of the Biology Division for publication in the open literature were published or are in press; in addition 9 ORNL reports were issued.

Listed below are the 192 papers; 3 issues of *Experimental Hematology;* 2 authored, 1 edited, and 4 coedited books; 2 edited Proceedings; the *Symposium on Molecular Aspects of Differentiation;* and 9 ORNL reports published during the period. Not listed are the 144 abstracts and 7 book reviews published, or the 223 items currently in press.

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Adler, H. I., C. E. Terry, and Alice A. Hardigree	Giant cells of *Escherichia coli*	J. Bacteriol., **95:** 139–142, 1968
Allen, R. C., J. W. Fisher, and Dorothy J. Moore	Preliminary purification of sheep and mouse erythropoietin by vertical flat bed discontinuous electrophoresis in acrylamide gel	Ann. N.Y. Acad. Sci., **149:** 63–70, 1968
Anderson, Louise, G. B. Price, and R. C. Fuller	Molecular diversity of the ribulose-1,5-diphosphate carboxylase from photosynthetic microorganisms	Science, **161:** 482–484, 1968
Andrews, G. A., C. C Congdon, C. L. Edwards, Nazareth Gengozian, Bill Nelson, and Helen Vodopick	Preliminary trials of clinical immunotherapy	Cancer Res., **26:** 2535–2541, 1967
Arnold, W. A., and J. R. Azzi	Chlorophyll energy levels and electron flow in photosynthesis	Proc. Natl. Acad. Sci. U.S., **61:** 29–35, 1968
Bender, M. A	Effects of radiation on chromosomes	Anais Acad. Brasil. Cienc. **39** (Suppl.): 77–93, 1967
	Somatic chromosomal aberrations: Use in the evaluation of human radiation exposures	Arch. Environ. Health, **16:** 556–564, 1968
Bender, M. A, and P. Carolyn Gooch	Cytogenetics methods: Somatic chromosome aberrations in occupationally irradiated humans	In *Accidental Irradiation at Place of Work* (Proceedings of the International Symposium held in Nice, France, from 26 to 29 April 1966). Euratom, 1967, pp. 133–139
Bender, M. A, P. Carolyn Gooch, and Sohei Kondo	The Gemini XI S-4 spaceflight-radiation interaction experiment: The human blood experiment	Radiation Res., **34:** 228–238, 1968
Bishop, B. S., and M. G. Hanna, Jr.	Computer Program for Inbred and/or Hybrid Animal Breeding and Production Colonies	ORNL-TM-2210
Bliss, Dorothy E., and Dorothy M. Skinner	VI. Animal energy exchange. 72. Oxygen consumption: Invertebrate tissues	In *Metabolism,* ed. by Philip L. Altman and Dorothy S. Dittmer. Federation of American Societies for Experimental Biology, Bethesda, Maryland, 1968, pp. 406–417

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Bosma, M. J., E. H. Perkins, and Takashi Makinodan	Further characterization of the lymphoid cell transfer system for the study of antigen-sensitive progenitor cells	J. Immunol., **101**: 963–972, 1968
Brewen, J. G., and R. D. Brock	The exchange hypothesis and chromosome-type aberrations	Mutation Res., 6: 245–255, 1968
Brick, J. O.	Specific-pathogen-free mice and their application to research	Med. Lab., **1**: 31–33 and 44, 1968
Brick, J. O., W. J. Roenigk, and G. P. Wilson	Chemotherapy of malignant lymphoma in dogs and cats	J. Am. Vet. Med. Assoc., **153**: 47–52, 1968
Brown, D. H., and G. David Novelli	Chromatographic differences between the cytoplasmic and mitochondrial tRNAs of *Neurospora crassa*	Biochem. Biophys. Res. Commun., **31**: 262–266, 1968
Brown, R. C., K. W. Cole, C. N. Cornell, and K. M. Brinkhous	Hemophilia-like disease of swine: Cytogenetic studies	Am. J. Vet. Res., **29**: 1491–1494, 1968
Buckton, Karin E., and Paul Nettesheim	*In vitro* and *in vivo* culture of mouse peripheral blood for chromosome preparations	Proc. Soc. Exptl. Biol. Med., **128**: 1106–1110, 1968
Clapp, N. K., A. W. Craig, and R. E. Toya, Sr.	Oncogenicity by methyl methanesulfonate in RF mice	Science, **161**: 913–914, 1968
	Pulmonary and hepatic oncogenesis during treatment of male RF mice with dimethylnitrosamine	J. Natl. Cancer Inst., **41**: 1213–1227, 1968
Cohen, Amikam, W. D. Fisher, Roy Curtiss III, and H. I. Adler	DNA isolated from *Escherichia coli* minicells mated with F^+ cells	Proc. Natl. Acad. Sci. U.S., **61**: 61–68, 1968
Cohn, W. E.	Introductory remarks by chairman	In *Advances in Tracer Methodology*, Vol. 4 (a collection of papers presented at the 11th Annual Symposium on Tracer Methodology), ed. by Seymour Rothchild. Plenum Press, New York, 1968, pp. 1–10
	The organization and functioning of biochemical nomenclature	J. Chem. Doc., 7: 72–73, 1967
Congdon, C. C	Foreword and Comment on Cover	Exptl. Hematol., **15**: iii, v–vi, 1968
	Foreword	Exptl. Hematol., **16**: iii, 1968
Congdon, C. C (editor)	Abstracts from the 10th Anniversary Bone Marrow Conference held November 17–18, 1967 at Oak Ridge, Tennessee (Alexander Hollaender, chairman); from the Workshop on Immunology of Leprosy held October 17, 1967 at Silver Spring, Maryland (C. C. Shepard, chairman); and one contributed paper	*Experimental Hematology No. 15*, Biology Division, Oak Ridge National Laboratory, 1968 (xi + 163 pp.)
	Abstracts from Bone Marrow Conference held April 17, 1968 in Atlantic City, New Jersey (N. B. Everett, chairman); from the Symposium on Myeloproliferative Disorders of Animals and Man (E. B. Howard and D. K. Merkeley, chairmen); and one contributed paper	*Experimental Hematology No. 16*, Biology Division, Oak Ridge National Laboratory, 1968 (x + 79 pp.)
	Abstracts from the Second International Conference on Germinal Centers of Lymphatic Tissue held June 26–28, 1968 at Padova, Italy (L. Fiore-Donati, chairman), and two contributed papers	*Experimental Hematology No. 17*, Biology Division, Oak Ridge National Laboratory, 1968 (xiv + 54 pp.)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Congdon, C. C, M. L. Simmons, and R. E. Toya	Reduced mortality from secondary disease after exposure to hypoxia	In *Advance in Transplantation, Proceedings of the First International Congress of the Transplantation Society,* ed. by J. Dausset, J. Hamburger, and G. Mathé. The Williams & Wilkins Company, Baltimore, 1968, pp. 437–439
Conger, A. D., and M. L. Randolph	Is age-dependent genetic damage in seeds caused by free radicals?	Radiation Botany, 8: 193–196, 1968
Cook, J. S., and Janet R. McGrath	Photoreactivating-enzyme activity in metazoa	Proc. Natl. Acad. Sci. U.S., **58:** 1359–1365, 1967
Cosgrove, G. E., W. B. Lushbaugh, Gretchen Humason, and M. G. Anderson	*Synhimantus* (Nematoda) associated with gastric squamous tumors in muskrats	Bull. Wildlife Disease Assoc., **4:** 54–57, 1968
Cosgrove, G. E., Bill Nelson, and Nazareth Gengozian	Helminth parasites of the tamarin, *Saguinus fuscicollis*	Lab. Animal Care, **18:** 654–656, 1968
	Parasites of the imported tamarin, *Saguinus fuscicollis*	ORAU-106; ORAU Medical Division Research Report for 1967, pp. 217–220
Cosgrove, G. E., H. E. Walburg, Jr., and A. C. Upton	Gastrointestinal lesions in aged conventional and germfree mice exposed to radiation as young adults	In *Gastrointestinal Radiation Injury* (Report of a Symposium held at Richland, Washington, September 25–28, 1966), ed. by M. F. Sullivan. Excerpta Medica Foundation, Amsterdam, 1968, pp. 303–312
Curtiss, Roy, III	Ultraviolet-induced genetic recombination in a partially diploid strain of *Escherichia coli*	Genetics, **58:** 9–54, 1968
Davidson, J. N., and W. E. Cohn (coeditors)	*Progress in Nucleic Acid Research and Molecular Biology,* Vol. 5 (450 pp.)	Academic Press, New York and London, 1966
	Progress in Nucleic Acid Research and Molecular Biology, Vol. 6 (430 pp.)	Academic Press, New York and London, 1967
	Progress in Nucleic Acid Research and Molecular Biology, Vol. 7 (467 pp.)	Academic Press, New York and London, 1967
	Progress in Nucleic Acid Research and Molecular Biology, Vol. 8 (389 pp.)	Academic Press, New York and London, 1968
Davis, M. L., A. C. Upton, G. E. Cosgrove, and Lou C. Satterfield	Effect of age of recipient mice on growth and differentiation of transplanted bone marrow cells	Proc. Soc. Exptl. Biol. Med., **128:** 1149–1153, 1968
de Serres, F. J.	Genetic analysis of the extent and type of functional inactivation in irreparable recessive lethal mutations in the *ad-3* region of *Neurospora crassa*	Genetics, **58:** 69–77, 1968
de Serres, F. J. (Principal Investigator)	Neurospora Experiment P-1037 Quarterly Progress Report to the National Aeronautics and Space Administration, December 16, 1966–March 15, 1967	ORNL-TM-2189
	Neurospora Experiment P-1037 Quarterly Progress Report to the National Aeronautics and Space Administration, March 16–June 30, 1967	ORNL-TM-2190
	Neurospora Experiment P-1037 Quarterly Progress Report to the National Aeronautics and Space Administration, July 1–September 30, 1967	ORNL-TM-2235
	Neurospora Experiment P-1037 Quarterly Progress Report to the National Aeronautics and Space Administration, October 1–December 31, 1967	ORNL-TM-2310

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
de Serres, F. J., and H. E. Brockman	Homology tests on presumed multilocus deletions in the *ad-3* region of *Neurospora crassa* induced by the acridine mustard ICR-170	Genetics, **58**: 79–83, 1968
de Serres, F. J., H. V. Malling, and B. B. Webber	Dose-rate effects on inactivation and mutation-induction in *Neurospora crassa*	In *Brookhaven Symposia in Biology, No. 20: Recovery and Repair Mechanisms in Radiobiology*. Brookhaven National Laboratory, Upton, New York, 1967, pp. 56–76
de Serres, F. J., and B. B. Webber	The combined effect of weightlessness and radiation on inactivation and mutation-induction in *Neurospora crassa* during the Biosatellite II mission	BioScience, **18**: 590–595, 1968
Dolin, M. I.	Kinetics of malic-lactic transhydrogenase. Effect of the keto-enol tautomerism of oxalacetate on the kinetics of oxalacetate formation and utilization	J. Biol. Chem., **243**: 3916–3923, 1968
Donnellan, J. E., Jr., J. L. Hosszu, R. O. Rahn, and R. S. Stafford	Effect of temperature on the photobiology and photochemistry of bacterial spores	Nature, **219**: 964–965, 1968
Donnellan, J. E., Jr., and R. S. Stafford	The ultraviolet photochemistry and photobiology of vegetative cells and spores of *Bacillus megaterium*	Biophys. J., **8**: 17–28, 1968
Dumont, J. N., and Everett Anderson	Vitellogenesis in the horseshoe crab, *Limulus polyphemus*	J. de Microscopie, **6**: 791–806, 1967
Dunaway, P. B., G. E. Cosgrove, and J. D. Story	Capillaria and trypanosoma infestations in *Microtus ochrogaster*	Bull. Wildlife Disease Assoc., **4**: 18–20, 1968
Ehling, U. H., R. B. Cumming, and H. V. Malling	Induction of dominant lethal mutations by alkylating agents in male mice	Mutation Res., **5**: 417–428, 1968
Endo, Eiji	Kidney lysozyme in hypoxic radiation chimeras	Exptl. Hematol., **15**: 158–161, 1968
Fisher, J. W., F. E. Hatch, B. L. Roh, R. C. Allen, and B. J. Kelley	Erythropoietin inhibitor in kidney extracts and plasma from anemic uremic human subjects	Blood, **31**: 440–452, 1968
Franklin, J. A., and C. B. Richter	The isolation of *Salmonella poona* and a nonmotile variant from laboratory mice	Lab. Animal Care, **18**: 92–93, 1968
Fujimura, R. K., and Elliot Volkin	Biochemical analysis of the naturally repaired sections of bacteriophage T5 deoxyribonucleic acid. I. Bromodeoxyuridine incorporation into parental deoxyribonucleic acid in the absence of deoxyribonucleic acid replication	Biochemistry, **7**: 3488–3498, 1968
Goodman, Joan Wright, and Sarah G. Shinpock	Influence of thymus cells on erythropoiesis of parental marrow in irradiated hybrid mice	Proc. Soc. Exptl. Biol. Med., **129**: 417–422, 1968
Goodman, Joan Wright, and H. B. Wheeler	Factors influencing growth of parental marrow grafts in irradiated F_1 hybrid mice	Transplantation, **6**: 173–186, 1968
	Factors that modify poor growth of parental marrow cells in F_1 hybrid mice	In *Advance in Transplantation, Proceedings of the First International Congress of The Transplantation Society*, ed. by J. Dausset, J. Hamburger, and G. Mathé. The Williams & Wilkins Company, Baltimore, 1968, pp. 427–430
Grell, E. H., K. Bruce Jacobson, and J. B. Murphy	Alterations of genetic material for analysis of alcohol dehydrogenase isozymes of *Drosophila melanogaster*	Ann. N. Y. Acad. Sci., **151**: 441–455, 1968
Grell, Rhoda F.	The relation between inversion heterozygosity and dominant lethality in *Drosophila pseudoobscura*	Am. Naturalist, **102**: 87–89, 1968
Gude, W. D.	*Autoradiographic Techniques: Localization of Radioisotopes in Biological Material* (xiii + 113 pp.)	Prentice-Hall, Inc., Englewood Cliffs, N.J., 1968

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Haber, A. H.	An examination of the concepts of "dormancy" and "germination" from studies with lettuce seeds	In *Physiology, Ecology, and Biochemistry of Germination,* Vol. I (Proceedings of the International Symposium held at the Botanic Insitute of the Ernst-Moritz-Arndt-University of Greifswald, 8th to 14th September 1963), ed. by Heinrich Borriss. Ernst-Moritz-Arndt-University, Greifswald, 1967, pp. 47–53
	Ionizing radiations as research tools	In *Annual Review of Plant Physiology,* Vol. 19, ed. by Leonard Machlis, and W. R. Briggs and R. B. Park (assoc. eds.). Annual Reviews, Inc., Palo Alto, California, 1968, pp. 463–489
Haber, A. H., and L. L. Triplett	Cancelling errors in the quantitative determination of α-amylase activity	ASB Bull., 15: 38, 1968
Hager, C. B., and F. T. Kenney	Regulation of tyrosine-α-ketoglutarate transaminase in rat liver. VII. Hormonal effects on synthesis in the isolated, perfused liver	J. Biol. Chem., **243:** 3296–3300, 1968
Hanna, M. G., Jr., Mary W. Francis, and Leona C. Peters	Localization of ^{125}I-labeled antigen in germinal centers of mouse spleen: Effects of competitive injection of specific on noncrossreacting antigen	Immunology, **15:** 75–91, 1968
Hanna, M. G., Jr., and A. K. Szakal	Localization of ^{125}I-labeled antigen in germinal centers of mouse spleen: Histologic and ultrastructural autoradiographic studies of the secondary immune reaction	J. Immunol., **101:** 949–962, 1968
Harrison, A. P., Jr., Vivian E. Raabe, and Gertrude H. Brummett	Detection of *in vivo* protein photooxidation by means of tritium loss from the histidine moieties	Proc. Soc. Exptl. Biol. Med., **127:** 538–543, 1968
Hartman, F. C.	Irreversible inactivation of triose phosphate isomerase by 1-hydroxy-3-iodo-2-propanone phosphate	Biochem. Biophys. Res. Commun., **33:** 888–894, 1968
Hellman, Alfred, D. H. Martin, and L. J. Wopschall	The influence of X-irradiation on survival and interferon levels in viral infected mice	Proc. Soc. Exptl. Biol. Med., **128:** 455–458, 1968
Hilse, K. M., and R. A. Popp	Gene duplication as the basis for amino acid ambiguity in the alpha-chain polypeptides of mouse hemoglobins	Proc. Natl. Acad. Sci. U. S., **61:** 930–936, 1968
Hollaender, Alexander	Foreword	In *Autoradiographic Techniques: Localization of Radioisotopes in Biological Material,* by W. D. Gude, Prentice-Hall, Inc., Englewood Cliffs, N.J., 1968, p. iii
	Foreword	In *International Symposium on Enzymatic Aspects of Metabolic Regulation* (held at Mexico City, Mexico, November 28–December 1, 1966), ed. by M. P. Stulberg. National Cancer Institute Monograph No. 27, U.S. Department of Health, Education, and Welfare; Public Health Service, National Cancer Institute, Bethesda, Maryland, 1967, pp. ix–x
	Introduction – *Symposium on Peaceful Uses of Atomic Radiation*	Anais Acad. Brasil. Cienc., **39** (Suppl.): 5–6, 1967
	Preface – *Basic Mechanisms in Photochemistry and Photobiology* (International Symposium held at Caracas, Venezuela, December 4–8, 1967) (in English, with Spanish translation)	Photochem. Photobiol., **7:** 511–514, 1968

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Jacobson, Ann B.	A procedure for isolation of proplastids from etiolated maize leaves	J. Cell Biology, 38: 238–244, 1968
Jacobson, Ann B., and R. B. Williams	Sedimentation studies on RNA from proplastids of *Zea mays*	Biochim. Biophys. Acta, **169**: 7–13, 1968
Jacobson, K. Bruce	Alcohol dehydrogenase of *Drosophila:* Interconversion of isoenzymes	Science, **159**: 324–325, 1968
Jared, D. W., and R. A. Wallace	Comparative chromatography of the yolk proteins of teleosts	Comp. Biochem. Physiol., **24**: 437–443, 1968
Jones, J. B., and M. L. Simmons	Innovar-vet® as an intramuscular anesthetic for rats	Lab. Animal Care, **18**: 642–643, 1968
Kenney, F. T., and W. L. Albritton	Repression of enzyme synthesis at the transitional level and its hormonal control	In *The Year Book of Cancer* (1966–1967 Year Book Series), ed. by R. L. Clark and R. W. Cumley. Year Book Medical Publishers, Inc., Chicago, 1967, pp. 394–397
Kenney, F. T., Darold Holten, and W. L. Albritton	Hormonal regulation of liver enzyme synthesis	In *International Symposium of Enzymatic Aspects of Metabolic Regulation* (held at Mexico City, Mexico, November 28–December 1, 1966), ed. by M. P. Stulberg. National Cancer Institute Monograph No. 27, U.S. Department of Health, Education, and Welfare; Public Health Service, National Cancer Institute, Bethesda, Maryland, 1967, pp. 315–323
Kenney, F. T., D. D. Holten, and C. B. Hager	Hormonal regulation of enzyme synthesis	In *Molecular Basis of Endocrinology* (Gunma Symposia on Endocrinology, Vol. 5), ed. by Nubuo Ui. Institute of Endocrinology, Gunma University, Maebashi, Japan, 1968, pp. 31–41
Khym, J. X., and Mayo Uziel	The use of the cetyltrimethylammonium cation in terminal sequence analyses of ribonucleic acids	Biochemistry, 7: 422–426, 1968
Kimball, R. F.	Symposium on Molecular Aspects of Differentiation (and Introduction)	J. Cellular Physiol., **72**, Suppl. 1, October 1968, 239 pp. incl. index
Kimball, R. F., and S. F. Carson	Biology Division Annual Progress Report for the Period Ending December 31, 1967	ORNL-4240
Knopp, J. A., and J. W. Longworth	Energy transfer in oligotyrosyl compounds: Fluorescence quenching as a function of the ionization of the phenolic hydroxyl groups	Biochim. Biophys. Acta, **154**: 436–443, 1968
Kretchmar, A. L., and W. R. Conover	Early proliferation of transplanted spleen colony forming cells	Proc. Soc. Exptl. Biol. Med., **129**: 218–220, 1968
Lindsley, D. L., and E. H. Grell (with Isabel L. Norton, ed., and Eileen J. Slaughter, consulting ed.)	*Genetic Variations of* Drosophila melanogaster	Carnegie Institution of Washington, Washington, D. C., 471 pp., illus., + preface and supplementary illus.
Lindsley, D. L., L. Sandler, Benedetto Nicoletti, and G. Trippa	Genetic control of recombination in *Drosophila*	In *Replication and Recombination of Genetic Material,* ed. by W. J. Peacock and R. D. Brock. Australian Academy of Science, Canberra, 1968, pp. 253–269
Lion, M. B.	Search for a mechanism for the increased sensitivity of 5-bromouracil-substituted DNA to ultraviolet radiation	Biochim. Biophys. Acta, **155**: 505–520, 1968
Liverman, J. L. (chairman), R. A. Popp (cochairman), N. G. Anderson (cochairman), and W. Boyle	Abstracts of the Conference on Viral, Tumor, and Transplantation Antigen Isolation, Oak Ridge National Laboratory, October 25–27, 1967	Printed in Vol. 6, No. 4 (July 1968 issue) of Transplantation, 53 pp. (632–684 incl.)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Longworth, J. W. (editor)	*Basic Mechanisms in Photochemistry and Photobiology* (International Symposium held at Caracas, Venezuela, December 4–8, 1967)	Printed as Vol. 7, No. 6 (June 1968 issue) of Photochem. Photobiol., 327 pp. (511–837 incl.)
Longworth, J. W.	Excited state interactions in macromolecules	Photochem. Photobiol., 7: 587–596, 1968
Malling, H. V., and F. J. de Serres	Correlation between base-pair transition and complementation pattern in nitrous acid-induced *ad-3B* mutants of *Neurospora crassa*	Mutation Res., **5**: 359–371, 1968
	Identification of genetic alterations induced by ethyl methanesulfonate in *Neurospora crassa*	Mutation Res., 6: 181–193, 1968
Manney, T. R.	Regulation of factors that influence the *in vitro* stability of tryptophan synthetase from yeast	J. Bacteriol., **96**: 403–408, 1968
Mattingly, Ellen, and Curtis Parker	Nucleic acid synthesis during larval development of *Rhynchosciara*	J. Insect Physiol., **14**: 1077–1083, 1968
	Sequence of puff formation in *Rhynchosciara* polytene chromosomes	Chromosoma, **23**: 255–270, 1968
Mazur, Peter	Factors affecting cell injury in cryosurgical freezing	Bull. Millard Fillmore Hosp., **14**: 123–128, 1967
	Physical-chemical factors underlying cell injury in cryosurgical freezing	In *Cryosurgery*, ed. by R. W. Rand, A. P. Rinfret, and Hans von Leden. Charles C Thomas, Springfield, Illinois, 1968, pp. 32–51
	Survival of fungi after freezing and desiccation: The fungal population	In *The Fungi: An Advanced Treatise*, Vol. III, ed. by G. C. Ainsworth and A. S. Sussman. Academic Press, New York and London, 1968, pp. 325–394
Mazur, Peter, and Janice J. Schmidt	Interactions of cooling velocity, temperature, and warming velocity on the survival of frozen and thawed yeast	Cryobiology, **5**: 1–17, 1968
McLeish, John	Chemical and autoradiographic studies of intranucleolar DNA in *Vicia faba*	Exptl. Cell Res., **51**: 157–166, 1968
Merriam, J. R.	The meiotic behavior of tandem acrocentric compound X chromosomes in *Drosophila melanogaster*	Genetics, **59**: 351–366, 1968
Modak, S. P., Georgia Morris, and Tuneo Yamada	DNA synthesis and mitotic activity during early development of chick lens	Develop. Biol., **17**: 544–561, 1968
Moseley, B. E. B., and Jane K. Setlow	Transformation in *Micrococcus radiodurans* and the ultraviolet sensitivity of its transforming DNA	Proc. Natl. Acad. Sci. U.S., **61**: 176–183, 1968
Nettesheim, Paul, and Anna S. Hammons	Lack of recruitment of antibody forming cells during the late haemagglutinin response	Nature, **220**: 297–299, 1968
Nettesheim, Paul, M. G. Hanna, Jr., and W. D. Fisher	Further studies on the effect of serum alpha macroglobulin on regeneration of hemopoietic tissue after X-irradiation	Radiation Res., **35**: 378–389, 1968
Nettesheim, Paul, and Takashi Makinodan	*In vivo* chamber culture technique	In *Methods in Developmental Biology*, ed. by F. H. Wilt and N. K. Wessells; general editor John P. Trinkaus. Thomas Y. Crowell Company, New York, 1967, pp. 471–483
Nettesheim, Paul, and Mary Lou Williams	Regenerative potential of immunocompetent cells. II. Factors influencing recovery of secondary antibody-forming potential from X-irradiation	J. Immunol., **100**: 760–770, 1968
Niyogi, S. K., and C. A. Thomas, Jr.	The stability of oligoadenylate-polyuridylate complexes as measured by thermal chromatography	J. Biol. Chem., **243**: 1220–1223, 1968

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Novelli, G. David	Amino acid incorporation in rat and trout liver	In *Trout Hepatoma Research Conference Papers – Research Report 70* (3rd National Trout Hepatoma Research Conference, Portland, Oregon, March 18–19, 1965), ed. by J. E. Halver and I. A. Mitchell. Bureau of Sport Fisheries and Wildlife, Washington, 1967, pp. 72–77
	Discussion	In *International Symposium on Enzymatic Aspects of Metabolic Regulation* (held at Mexico City, Mexico, November 28–December 1, 1966), ed. by M. P. Stulberg. National Cancer Institute Monograph No. 27, U.S. Department of Health, Education, and Welfare; Public Health Service, National Cancer Institute, Bethesda, Maryland, 1967, pp. 325–327
Oakberg, E. F.	^{3}H-uridine labeling of mouse oocytes	Arch. Anat. Microscop. Morphol. Exp., **56**: 171–184, 1967
	Mammalian gametogenesis and species comparisons in radiation response of the gonads	In *Effects of Radiation on Meiotic Systems* (Report of a Study Group on the Effects of Radiation on Meiotic Systems, organized by the IAEA, held in Vienna, Austria, 8–11 May 1967). International Atomic Energy Agency, Vienna, 1968, pp. 3–15
	Relationship between stage of follicular development and RNA synthesis in the mouse oocyte	Mutation Res., **6**: 155–165, 1968
Oakberg, E. F., and Ann M. Hughes	Deuterium oxide effect on spermatogenesis in the mouse	Exptl. Cell Res., **50**: 306–314, 1968
Odell, T. T., Jr., and C. W. Jackson	Polyploidy and maturation of rat megakaryocytes	Blood, **32**: 102–110, 1968
Palmer, Winifred G., and John Papaconstantinou	Biochemistry of bovine lens proteins. III. Chemical and physical properties of α-crystallin subunits	Biochemistry, 7: 243–253, 1968
	Increase in the complexity of α-crystallins during differentiation of lens cells	Nature, **219**: 170–172, 1968
Papaconstantinou, John, and Emilia M. Julku	The regulation of ribosomal RNA synthesis and ribosomal assembly in the vertebrate lens	J. Cellular Physiol., 72 (Suppl. 1): 161–179, 1968
Parker, D. R.	A survey of methods for the induction of aberrations in meiotic stages in *Drosophila* females and for observation of their disjunctional properties in the ensuing meiotic divisions	In *Effects of Radiation on Meiotic Systems* (Report of a Study Group on the Effects of Radiation on Meiotic Systems, organized by the IAEA, held in Vienna, Austria, 8–11 May 1967). International Atomic Energy Agency, Vienna, 1968, pp. 209–218
Pavan, Crodowaldo, and A. L. P. Perondini	Heterozygous puffs and bands in *Sciara ocellaris* Comstock (1882)	Exptl. Cell Res., **48**: 202–205, 1967
Paxon, C. L., Jr., and L. H. Smith	Blood volume of the mouse	Exptl. Hematol., **17**: 42–47, 1968
Pearlstein, R. M.	Fluorescence excitation of chlorophyll in green plants by β particles	Biochim. Biophys. Acta, **153**: 504–507, 1968
	Possibility of limited pairon condensation in double-stranded DNA	Phys. Rev. Letters, **20**: 594–596, 1968

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Popp, R. A., and N. G. Anderson	Foreword – Conference on Viral, Tumor, and Transplantation Antigen Isolation (held at Oak Ridge, Tennessee, October 25–27, 1967)	Transplantation, **6**: 632, 1968
Rahn, R. O., and J. L. Hosszu	Photochemistry of polynucleotides: A summary of temperature effects	Photochem. Photobiol., 7: 637–642, 1968
	Photoproduct formation in DNA at low temperatures	Photochem. Photobiol., **8**: 53–63, 1968
Randolph, M. L., J. A. Heddle, and J. L. Hosszu	Dependence of ESR signals in seeds on moisture content	Radiation Botany, **8**: 339–343, 1968
Randolph, M. L., Marvin Slater, A. C. Upton, and J. W. Conklin	Late effect of fast neutrons and gamma rays in mice as influenced by the dose rate of irradiation: Methodology of irradiation and dosimetry	Radiation Res., **32**: 475–492, 1967
Ray, P. M.	Autoradiographic study of cell wall deposition in growing plant cells	J. Cell Biol., **35**: 659–674, 1967
Reel, J. R., and F. T. Kenney	"Superinduction" of tyrosine transaminase in hepatoma cell cultures: Differential inhibition of synthesis and turnover by actinomycin D	Proc. Natl. Acad. Sci. U.S., **61**: 200–206, 1968
Regan, J. D.	Evidence for photoreactivation in marine fish cells *in vitro*	In *in vitro,* Vol. III – *Differentiation and Defense Mechanisms in Lower Organisms,* ed. by M. M. Sigel. Distributed for The Tissue Culture Association, Inc., by The Williams and Wilkins Company, Baltimore, 1968, pp. 166–168
Regan, J. D., and J. S. Cook	Photoreactivation in an established vertebrate cell line	Proc. Natl. Acad. Sci. U.S., **58**: 2274–2279, 1967
Regan, J. D., J. S. Cook, and W. H. Lee	Photoreactivation of amphibian cells in culture	J. Cell. Physiol., **71**: 173–176, 1968
Regan, J. D., M. M. Sigel, W. H. Lee, K. A. Llamas, and Annie R. Beasley	Chromosomal alterations in marine fish cells *in vitro*	Can. J. Genet. Cytol., **10**: 448–453, 1968
Regan, J. D., J. E. Trosko, and W. L. Carrier	Evidence for excision of ultraviolet-induced pyrimidine dimers from the DNA of human cells *in vitro*	Biophys. J., **8**: 319–325, 1968
Riggsby, W. S., and Virginia Merriam	RNA-DNA hybridization: Demonstration in a mammalian system of competition by preincubation	Science, **161**: 570–571, 1968
Rogers, Stanfield, and Peter Pfuderer	The use of viruses as carriers of added genetic information. I. The isolation of polylysine induced by polyadenine-modified tobacco mosaic viral RNA in tobacco plants	Nature, **219**: 749–751, 1968
Rosen, Lawrence	Ethylation *in vivo* of purines in rat-liver tRNA by L-ethionine	Biochem. Biophys. Res. Commun., **33**: 546–550, 1968
Rosen, Lawrence, E. L. Murray, and G. David Novelli	Cell-free incorporation of amino acids by a trout-liver system. Heat-labile aminoacyl transferase activity	Can. J. Biochem., **45**: 2005–2014, 1967
Rudack, Diana, and R. A. Wallace	On the site of phosvitin synthesis in *Xenopus laevis*	Biochim. Biophys. Acta, **155**: 299–301, 1968
Russell, Liane B.	The use of sex-chromosome anomalies for measuring radiation effects in different germ-cell stages of the mouse	In *Effects of Radiation on Meiotic Systems* (Report of a Study Group on the Effects of Radiation on Meiotic Systems, organized by the IAEA, held in Vienna, Austria, 8–11 May 1967). International Atomic Energy Agency, Vienna, 1968, pp. 27–41

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Russell, W. L.	Factors that affect the radiation induction of mutations in the mouse	Anais Acad. Brasil. Cienc., **39** (Suppl.): 65–75, 1967
	Recent studies on the genetic effects of radiation in mice	Pediatrics, **41**: 223–230, 1968
	Repair mechanisms in radiation mutation induction in the mouse	In *Brookhaven Symposia in Biology, No. 20: Recovery and Repair Mechanisms in Radiobiology.* Brookhaven National Laboratory, Upton, New York, 1967, pp. 179–189
Sassen, A. E., E. H. Perkins, and R. A. Brown	Immunogenic potency of human γ-globulin in mice	Immunology, **14**: 247–256, 1968
Setlow, Jane K., and F. J. Bollum	The minimum size of the substrate for yeast photoreactivating enzyme	Biochim. Biophys. Acta, **157**: 233–237, 1968
Setlow, Jane K., Dianne C. Brown, M. E. Boling, Alice A. Mattingly, and M. P. Gordon	Repair of deoxyribonucleic acid in *Hemophilus influenzae*. 1. X-ray sensitivity of ultraviolet-sensitive mutants and their behavior as hosts to ultraviolet-irradiated bacteriophage and transforming deoxyribonucleic acid	J. Bacteriol., **95**: 546–558, 1968
Setlow, R. B.	The photochemistry, photobiology, and repair of polynucleotides	In *Progress in Nucleic Acid Research and Molecular Biology*, Vol. 8, ed. by J. N. Davidson and W. E. Cohn. Academic Press, New York and London, 1968, 257–295
	Photoproducts in DNA irradiated *in vivo*	Photochem. Photobiol., **7**: 643–649, 1968
	Steps in the repair of DNA: Fact and fancy	In *Brookhaven Symposia in Biology, No. 20: Recovery and Repair Mechanisms in Radiobiology.* Brookhaven National Laboratory, Upton, New York, 1967, pp. 1–16
Setlow, R. B., and W. L. Carrier	The excision of pyrimidine dimers *in vivo* and *in vitro*	In *Replication and Recombination of Genetic Material*, ed. by W. J. Peacock and R. D. Brock. Australian Academy of Science, Canberra, 1968, pp. 134–141
Shepard, C. C., and C. C Congdon	Increased growth of *Mycobacterium leprae* in thymectomized-irradiated mice after foot pad inoculation	Intern. J. Leprosy, **36**: 224–227, 1968
Shugart, Lee, Barbara H. Chastain, G. David Novelli, and M. P. Stulberg	Restoration of aminoacylation activity of undermethylated transfer RNA by *in vitro* methylation	Biochem. Biophys. Res. Commun., **31**: 404–409, 1968
Shugart, Lee, G. David Novelli, and M. P. Stulberg	Isolation and properties of undermethylated phenylalanine transfer ribonucleic acids from a relaxed mutant of *Escherichia coli*	Biochim. Biophys. Acta, **157**: 83–90, 1968
Simmons, M. L., and J. A. Franklin	A summer training program for veterinary students in laboratory animal medicine at Oak Ridge National Laboratory	J. Am. Vet. Med. Assoc., **148**: 1446, 1966
Simmons, M. L., C. B. Richter, J. A. Franklin, and R. W. Tennant	Prevention of infectious diseases in experimental mice	Proc. Soc. Exptl. Biol. Med., **126**: 830–837, 1967
Simmons, M. L., C. B. Richter, R. W. Tennant, and J. A. Franklin	Production of specific pathogen-free rats in plastic germfree isolator rooms	In *Advances in Germfree Research and Gnotobiology* (including the Proceedings of the International Symposium on Life Germfree Research, Nagoya and Inuyama, Japan, 1967), ed. by M. Miyakawa and T. D. Luckey. CRC Press (A Division of the Chemical Rubber Co.), Cleveland, Ohio, 1968, pp. 38–47

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Simmons, M. L., D. M. Robie, J. B. Jones, and L. J. Serrano	Effect of a filter cover on temperature and humidity in a mouse cage	Lab. Animals, **2**: 113–120, 1968
Simmons, M. L., and L. H. Smith	An anesthetic unit for small laboratory animals	J. Appl. Physiol., **25**: 324–325, 1968
Skinner, Dorothy M.	Isolation and characterization of ribosomal ribonucleic acid from the crustacean, *Gecarcinus lateralis*	J. Exptl. Zool., **169**: 408–418, 1968
Smith, L. H.	An *in vitro* bone marrow system for studying recovery from radiation injury	Bull. Inst. Nucl. Sci. "Boris Kidrich" (Belgrade), **17** (Suppl.): 17–19, 1967
Smith, L. H., and C. C Congdon	Biological effects of ionizing radiation	In *Human Transplantation,* ed. by F. T. Rapaport and J. Dausset. Grune & Stratton, Inc., New York, 1968, pp. 510–525
Smith, L. H., and T. W. McKinley, Jr.	Influence of temperature on X-ray sensitivity of mouse bone marrow cells *in vitro*	Radiation Res., **32**: 441–451, 1967
Sproston, Thomas, and R. B. Setlow	Ergosterol and substitutes for the ultraviolet radiation requirement for conidia formation in *Stemphylium solani*	Mycologia, 60: 104–114, 1968
Stafford, R. S., and J. E. Donnellan, Jr.	Photochemical evidence for conformation	Proc. Natl. Acad. Sci. U.S., **59**: 822–829, 1968
Stevens, Audrey, and S. K. Niyogi	Hydrolysis of oligoribonucleotides by an enzyme fraction from *Escherichia coli*	Biochem. Biophys. Res. Commun., **29**: 550–555, 1967
Stine, G. J.	Effect of storage in water on germination and enzyme activities	Neurospora Newsletter, **11**: 7–8, 1967
	Enzyme activities during the asexual cycle of *Neurospora crassa.* II. NAD- and NADP-dependent glutamic dehydrogenases and nicotinamide adenine dinucleotidase	J. Cell. Biol., **37**: 81–88, 1968
Stulberg, M. P.	Discussion	In *International Symposium on Enzymatic Aspects of Metabolic Regulation* (held at Mexico City, Mexico, November 28–December 1, 1966), ed. by M. P. Stulberg. National Cancer Institute Monograph No. 27, U.S. Department of Health, Education, and Welfare; Public Health Service, National Cancer Institute, Bethesda, Maryland, 1967, pp. 193–194
Stulberg, M. P. (editor)	*International Symposium on Enzymatic Aspects of Metabolic Regulation* (held at Mexico City, Mexico, November 28 to December 1, 1966)	National Cancer Institute Monograph No. 27, U.S. Department of Health, Education, and Welfare; Public Health Service, National Cancer Institute, Bethesda, Maryland, 1967, xiii + 343 pp.
Sutherland, Betsy M., W. L. Carrier, and R. B. Setlow	Photoreactivation *in vivo* of pyrimidine dimers in *Paramecium* DNA	Science, **158**: 1699–1700, 1967
	Pyrimidine dimers in the DNA of *Paramecium aurelia*	Biophys. J. **8**: 490–499, 1968
Szakal, A. K., and M. G. Hanna, Jr.	The ultrastructure of antigen localization and virus-like particles in mouse spleen germinal centers	Exptl. Mol. Pathol., **8**: 75–89, 1968
ten Bosch, J. J., R. O. Rahn, J. W. Longworth, and R. G. Shulman	Energy transfer in poly-L-tyrosine as a function of the degree of ionization of the phenolic hydroxyls. II. Electron spin resonance study of the triplet state	Proc. Soc. Natl. Acad. Sci. U.S., **59**: 1003–1008, 1968

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Upton, A. C.	Comparative observations on radiation carcinogenesis in man and animals	In *Carcinogenesis: A Broad Critique, A collection of papers presented at the Twentieth Annual Symposium on Fundamental Cancer Research, 1966.* The Williams & Wilkins Company, Baltimore, 1967, pp. 631–675
	The influence of dose rate in mammalian radiation biology: Quality effects	In *Dose Rate in Mammalian Radiation Biology* (proceedings of a Symposium on Dose Rate in Mammalian Radiation Biology held April 29–May 1, Oak Ridge, Tennessee), ed. by D. G. Brown, R. G. Cragle, and T. R. Noonan. USAEC, Division of Technical Information, 1968, pp. 22.1–22.18
	Radiation and carcinogenesis	Anais Acad. Brasil. Cienc., **39** (Suppl): 129–161, 1967
	Radiation carcinogenesis	In *Methods in Cancer Research,* Vol. IV, ed. by Harris Busch. Academic Press, Inc., New York and London, 1968, pp. 53–82
	The role of radiation in the etiology of leukemia	In *Proceedings of the International Conference on Leukemia-Lymphoma,* ed. by C. J. D. Zarafonetis. Lea & Febiger, Philadelphia, 1968, pp. 55–71
Upton, A. C., and G. E. Cosgrove	Radiation-induced leukemia	In *Experimental Leukemia,* ed. by Marvin A. Rich. Appleton-Century-Crofts, New York, 1968, pp. 131–158
Upton, A. C., and R. F. Kimball	Radiation biology	In *Principles of Radiation Protection – A Textbook of Health Physics,* ed. by K. Z. Morgan and J. E. Turner. John Wiley & Sons, Inc., New York, London, Sydney, 1967, pp. 398–447
Upton, A. C., M. L. Randolph, and J. W. Conklin	Late effects of fast neutrons and gamma rays in mice as influenced by the dose rate of irradiation: Life shortening	Radiation Res., 32: 493–509, 1967
Volkin, Elliot	Enzyme induction by bacteriophage	In *International Symposium on Enzymatic Aspects of Metabolic Regulation* (held at Mexico City, Mexico, November 28–December 1, 1966), ed. by M. P. Stulberg. National Cancer Institute Monograph No. 27, U.S. Department of Health, Education, and Welfare; Public Health Service, National Cancer Institute, Bethesda, Maryland, 1967, pp. 199–210
von Borstel, R. C.	Control of adult pests by the irradiation-of-male method	In *Isotopes and Radiation in Entomology* (Proceedings of an IAEA-FAO Symposium, Vienna, 4–8 December 1967). International Atomic Energy Agency, Vienna, Austria, 1968, pp. 331–337

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
von Borstel, R. C. (Principal Investigator)	(Habrobracon Experiment P-1079) Quarterly Progress Report to the National Aeronautics and Space Administration, April 1–June 30, 1967	ORNL-TM-2191
	Habrobracon Experiment P-1079 Quarterly Progress Report to the National Aeronautics and Space Administration, July 1–September 30, 1967	ORNL-TM-2311
	Habrobracon Experiment P-1079 Quarterly Progress Report to the National Aeronautics and Space Administration, October 1–December 31, 1967	ORNL-TM-2312
von Borstel, R. C., R. H. Smith, D. S. Grosch, Anna R. Whiting, R. L. Amy, M. B. Baird, P. D. Buchanan, Katherine T. Cain, Ruth Ann Carpenter, A. M. Clark, A. C. Hoffman, Martha S. Jones, Sohei Kondo, Margaret J. Lane, T. J. Mizianty, Mary Lou Pardue, Joan W. Reel, Diana B. Smith, Judith A. Steen, Julie T. Tindall, and L. R. Valcovic	Mutational response of *Habrobracon* in the Biosatellite II experiment	BioScience, **18**: 598–601, 1968
Walburg, H. E., Jr., G. E. Cosgrove, and A. C. Upton	Influence of microbial environment on development of myeloid leukemia in X-irradiated RFM mice	Intern. J. Cancer, **3**: 150–154, 1968
Wallace, R. A., and J. N. Dumont	The induced synthesis and transport of yolk proteins and their accumulation by the oocyte in *Xenopus laevis*	J. Cellular Physiol. **72** (Suppl. 1): 73–89, 1968
Wallace, R. A., and D. W. Jared	Estrogen induces lipophosphoprotein in serum of male *Xenopus laevis*	Science, **160**: 91–92, 1968
	Studies on amphibian yolk. VII. Serum phosphoprotein synthesis by vitellogenic females and estrogen-treated males of *Xenopus laevis*	Can. J. Biochem., **46**: 954–959, 1968
Waters, L. C., and G. David Novelli	The early change in *E. coli* leucine tRNA after infection with bacteriophage T2	Biochem. Biophys. Res. Commun., **32**: 971–976, 1968
Whiting, Anna R., R. H. Smith, and R. C. von Borstel	Methods for radiation studies during oogenesis in *Habrobracon juglandis* (Ashmead)	In *Effects of Radiation on Meiotic Systems* (Report of a Study Group on the Effects of Radiation on Meiotic Systems, organized by the IAEA, held in Vienna, Austria, 8–11 May 1967). International Atomic Energy Agency, Vienna, 1968, pp. 201–208
Whiting, P. W.	The chromosomes of *Mormoniella*	J. Heredity, **59**: 19–22, 1968
Whitson, G. L., A. A. Francis, and W. L. Carrier	Ultraviolet-induced pyrimidine dimers in *Tetrahymena*. I. Removal in the dark	Biochim. Biophys. Acta, **161**: 285–290, 1968
Wicks, W. D.	Induction of tyrosine-α-ketoglutarate transaminase in fetal rat liver	J. Biol. Chem., **243**: 900–906, 1968
	Tyrosine-α-ketoglutarate transaminase: Induction by epinephrine and adenosine-3′,5′-cyclic phosphate	Science, **160**: 997–998, 1968
Williams, G. R., and G. David Novelli	Ribosome changes following illumination of dark-grown plants	Biochim. Biophys. Acta, **155**: 183–192, 1968
Wust, C. J.	Binding of ^{125}I-labeled anti-L chain to polysomes of rat spleen *in vitro*	Arch. Biochem. Biophys., **126**: 524–529, 1968
Yamada, Tuneo, and Marion E. Roesel	Labeling of lens regenerate cells grafted into the newt optic chamber	Exptl. Cell Res., **50**: 649–652, 1968

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Yanders, A. F., J. G. Brewen, W. J. Peacock, and D. J. Goodchild	Meiotic drive and visible polarity in *Drosophila* spermatocytes	Genetics, **59:** 245–253, 1968
Yang, Wen-Kuang, and G. David Novelli	Isoaccepting tRNA's in mouse plasma cell tumors that synthesize different myeloma protein	Biochem. Biophys. Res. Commun., **31:** 534–539, 1968
	Multiple isoaccepting transfer RNA's in a mouse plasma cell tumor	Proc. Natl. Acad. Sci. U.S., **59:** 208–215, 1968
	Studies on the multiple isoaccepting transfer ribonucleic acids in mouse plasma cell tumors	In *Nucleic Acids in Immunology,* ed. by Otto J. Plescia and Werner Braun. Springer-Verlag New York Inc., 1968, pp. 644–659
Zeigel, R. F., R. L. Tyndall, T. E. O'Connor, Ernestine Teeter, and Barbara V. Allen	Observations on the morphology of a murine leukemia virus (Rauscher) propagated in tissue culture	In *Conference on Murine Leukemia,* ed. by M. A. Rich and J. B. Moloney. National Cancer Institute Monograph No. 22, U.S. Department of Health, Education, and Welfare; Public Health Service, Bethesda, Maryland, 1966, pp. 237–263

LECTURES

During 1968, members of the Biology Division presented 493 lectures and speeches in the United States and abroad. Included were papers given at professional society meetings; lectures and seminars given at the invitation of universities, colleges, scientific organizations and institutions, and professional groups or clubs; and lectures given as part of courses or under the Traveling Lecture program.

For details on speeches given outside the continental United States, see the section **Foreign Travel**; educational activities are described in the section on that subject (see Contents).

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Adler, H. I.	Cell division in *Escherichia coli*	Princeton University Conference on The Structure and Function of Cell Envelopes, Princeton, New Jersey
	Control of growth and division in bacteria	University of Puerto Rico, Rio Piedras
	Genetic control of cell growth and division in bacteria	(1) Iowa State University, Ames (2) Washington University, St. Louis, Missouri
	Genetic control of cell growth and division in *Escherichia coli*	(1) Cornell University, Ithaca, New York (2) Syracuse University, Syracuse, New York (3) Cellular Radiology Section, Los Alamos Scientific Laboratory, Los Alamos, New Mexico
Allen, R. C.	Quantitation of plasma and tissue esterase isoenzymes: Application to the study of physiological and pathological changes	(1) University of Washington, Seattle (2) University of California at Davis, Sacramento
Allen, R. C. (Dorothy J. Moore, and R. L. Tyndall)	Quantitation of plasma and tissue esterase isoenzymes: Application to the study of physiological and pathological changes (In *Third International Congress of Histochemistry and Cytochemistry,* Springer-Verlag New York Inc., 1968, pp. 5–6)	IIIrd International Congress of Histochemistry and Cytochemistry, New York City

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Anderson, Louise	Light and metabolites; control of the Calvin cycle in *Rhodospirillum rubrum*	(1) University of Tennessee, Knoxville (2) Western Michigan University, Kalamazoo (3) Rutgers University, New Brunswick, New Jersey
Anderson, Louise (and R. C. Fuller)	Energy metabolism and control of an enzyme of the Calvin cycle	1st International Congress of Photosynthesis, Freudenstadt, Germany
	Modulation of *Rhodospirillum rubrum* ribose 5-P isomerase [Plant Physiol., **43** (Suppl.): S-30, 1968]	American Society of Plant Physiologists, Amherst, Massachusetts
Anderson, R. E. (R. B. Cumming, and Fred Snyder)	The lipid composition of L-M cells grown in suspension culture	American Oil Chemists' Society, New York City
Arnold, W. A.	Photosynthesis	Charles F. Kettering Research Laboratory, Yellow Springs, Ohio
Bahner, C. T. (and D. G. Doherty)	Fate of 1-(4-dimethylaminobenzylidene)-indene in rats (Proc. Am. Assoc. Cancer Res., 9: 4, 1968)	American Association for Cancer Research (59th Annual Meeting), Atlantic City, New Jersey
Barnett, W. E.	Mitochondrial protein synthesis	Johns Hopkins University, Baltimore, Maryland
	Molecular biology of nucleic acids (series of 6 lectures)	University of Puerto Rico, San Juan
Barnett, W. E. (and J. L. Epler)	The genetic translational apparatus of mitochondria	Florida State University Alumni Studies Conference on Molecular Biology, Tallahassee
Bender, M. A	Radiation-induced chromosome aberrations (In *Proceedings of the XII International Congress of Genetics, Tokyo, Japan, August 19–28, 1968; Vol. II – Abstracts of Plenary Lectures, Invited Lectures, and Small Symposia.* The Science Council of Japan, Tokyo, 1968, p. 188)	(1) XIIth International Congress of Genetics, Tokyo, Japan (2) Symposium on Chromosomes and Related Problems, Sapporo, Hokkaido, Japan
Bender, M. A (and M. A. Barcinski) (read by J. G. Brewen)	Kinetics of two-break aberration production by X rays in human leukocytes	VIIth Conference on Mammalian Cytology and Somatic Cell Genetics, Gatlinburg, Tennessee
Bender, M. A (P. Carolyn Gooch, and Sohei Kondo)	Human blood experiments in the Gemini series	International Symposium on Genetic Effects of Space Environment, Tokyo, Japan
Brewen, J. G.	(1) Autoradiographic studies on the nature and behavior of chromosomal subunits (2) The nature and repair of radiation-induced chromosomal lesions	Cornell University, Ithaca, New York
Brewen, J. G. (and W. J. Peacock)	Dissimilarity of chromosome subunits involved in sister strand exchange (Genetics, 60: 211, 1968)	Genetics Society of America (37th Annual Meeting), Boston, Massachusetts
Brick, J. O.	Operation and requirements for barrier-type animal facilities	Symposium for Planning and Design of Animal Facilities for Biological Research, Ohio State University College of Medicine, Columbus
Brown, R. C.	Ultrastructure of murine myxosarcomas associated with Rauscher virus transformation	Oak Ridge Associated Universities, Oak Ridge, Tennessee
Brown, R. C. (and H. C. Hopps)	The development of a reliable Gram stain for bacteria in tissue sections	Tennessee Society of Pathologists and the Tennessee Medical Association, Chattanooga

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Caro, L. G.	Chromosome replication in *Escherichia coli*	(1) Stanford University, Stanford, California (2) University of California, Los Angeles (3) California Institute of Technology, Pasadena (4) Scripps Institute, University of California, La Jolla (5) University of Chicago, Chicago, Illinois (6) Kansas State University, Manhattan (7) University of Tennessee, Knoxville
Caro, L. G. (and Claire M. Berg)	Chromosome replication in some K-12 strains of *Escherichia coli*	Cold Spring Harbor Symposium on DNA Replication, Repair, and Recombination, Cold Spring Harbor, New York
Carrier, W. L. (and R. B. Setlow)	Enzymes involved in dimer excision (Abstr. of 5th Intern. Congr. Photobiol., 1968, p. Hf-7)	Fifth International Congress of Photobiology, Hanover, New Hampshire
Chu, E. H. Y.	Genetic studies of mammalian cells in culture	University of Toledo, Toledo, Ohio
	Mutational studies of mammalian somatic cells in culture	(1) University of North Carolina School of Medicine, Chapel Hill (2) Oak Ridge Associated Universities, Oak Ridge, Tennessee
	Quantitative cytogenetic analysis of mammalian cells	Summer Science Seminar, a course sponsored by the Chinese National Research Council, Taipei, Taiwan, China
	Spontaneous and induced mutations in Chinese hamster cells in culture	Symposium on Genetics (under the joint auspices of the Sino-American Cooperative Committee, the Academia Sinica, and the U.S. National Academy of Sciences), Taipei, Taiwan, China
Chu, E. H. Y. (and H. V. Malling)	Chemical mutagenesis in Chinese hamster cells *in vitro* (In *Proceedings of the XII International Congress of Genetics, Tokyo, Japan, August 19–28, 1968; Vol. I – Abstracts of Contributed Papers.* The Science Council of Japan, Tokyo, Japan, 1968, p. 102)	XIIth International Congress of Genetics, Tokyo, Japan
Clapp, N. K.	(1) Has science made Bible Christianity obsolete? (2) Science, the scientist, and the Gospel (3) Bible creation vs. evolution (4) I propose restructure of the church	Milligan College, Johnson City, Tennessee
	Radiation and nitrosamine carcinogenesis	Illinois Institute of Technology Research, Chicago
Clapp, N. K. (and A. W. Craig)	Differences in tumor cell types and organ sites in RF mice treated with dimethylnitrosamine (DMN) and diethylnitrosamine (DEN) (Proc. Am. Assoc. Cancer Res., 9: 14, 1968)	American Association for Cancer Research (59th Annual Meeting), Atlantic City, New Jersey
Clapp, N. K. (E. B. Darden, Jr., R. S. Bender, and M. C. Jernigan)	Preliminary data on the relative biological effectiveness (RBE) of 60-Mev protons for life-shortening in RF mice (Radiation Res., 35: 556, 1968)	Radiation Research Society (16th Annual Meeting), Houston, Texas

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Cohen, Amikam	Properties of DNA isolated from *E. coli* minicells following mating	University of Wisconsin, Madison
Cohen, Amikam (W. D. Fisher, Roy Curtiss III, and H. I. Adler)	The properties of DNA transferred to minicells during conjugation	Cold Spring Harbor Symposium on DNA Replication, Repair, and Recombination, Cold Spring Harbor, New York
Cohn, W. E.	Problems in representing polymers of biological importance (Abstr. of papers, 156th Meet., Am. Chem. Soc., 1968, Poly. No. 57)	American Chemical Society (156th National Meeting), Atlantic City, New Jersey
Congdon, C. C	(1) Bone marrow transplantation (2) Pathological processes	University of North Carolina, Greensboro
	Germinal centers, Hassall's corpuscles, and epithelioid granulation tissue (Exptl. Hematol., 17: 38–39, 1968)	Second International Conference on Germinal Centers of Lymphatic Tissue, Padova, Italy
	Secondary disease in radiation chimeras	(1) CNEN-Euratom Immunogenetics Group, Casaccia, Rome, Italy (2) IAEA Panel Meeting on Bone Marrow Transplantation, Moscow, Russia
Conte, F. P.	Cellular and subcellular aspects of gill differentiation	U.S.-Japan Symposium on Endocrine Glands and Osmoregulation in Fishes, Tokyo, Japan
	Comparative biochemistry of the gill	Symposium on Fish in Basic Research, University of South Dakota, Vermillion
	Succinic dehydrogenase activity from gill mitochondria isolated from three species of euryhaline fishes	American Association for the Advancement of Science (Am. Soc. Zool.), Dallas, Texas
Cook, J. S.	Repair of DNA by photoreactivation	University of Massachusetts, Amherst
Cook, J. S. (and A. J. Zelnis)	Labeling of DNA bases and their photoproducts with ^{3}H-dimethyl sulfate after ultraviolet irradiation (Abstr. of 5th Intern. Congr. Photobiol., 1968, p. Eh-8)	Fifth International Congress of Photobiology, Hanover, New Hampshire
Cosgrove, G. E.	Etiology of diseases in lower animals	UT-AEC Agricultural Research Laboratory Seminar, Oak Ridge, Tennessee
	(1) Mammalian radiobiology (2) Radiation protection and recovery	Radiation Biology Institute, Wayne State University, Detroit, Michigan
	Radiation biology: Chronic, somatic, genetic	ORAU Health Physics Certification Training Course, Oak Ridge, Tennessee
	The value of pathology in laboratory animal disease problems	American Academy of Laboratory Animal Science (East Tennessee Branch), Oak Ridge
Cumming, R. B.	Fate and metabolism of some mutagenic alkylating agents in male mice (Genetics, 60: 170–171, 1968)	Genetics Society of America (37th Annual Meeting), Boston, Massachusetts
Cumming, R. B. (Kiki B. Hellman, Alfred Hellman, and G. David Novelli)	H^{3}-uridine incorporation in LM cells *in vivo* and after return to culture	Tissue Culture Association (19th Annual Meeting), San Juan, Puerto Rico

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Curtiss, Roy, III	(1) Bacterial conjugation with F^+ donors of *Escherichia coli* (2) Bacterial conjugation with Hfr donors of *Escherichia coli* (3) Chromosome structure and replication in *Escherichia coli*	Cornell University, Ithaca, New York
	Requirement for effective homologous pairing to obtain genetic transfer during conjugation in *Escherichia coli* K-12 (Genetics, 60: 171, 1968)	Genetics Society of America (37th Annual Meeting), Boston, Massachusetts
	Studies on bacterial conjugation	(1) Swarthmore College, Swarthmore, Pennsylvania (2) Yale University, New Haven, Connecticut
Curtiss, Roy, III (L. J. Charamella, D. R. Stallions, and J. A. Mays)	Role of the F^- recipient during conjugation with Hfr, F^+, and F' donors of *Escherichia coli* K-12	Symposium on Entry and Control of Foreign Nucleic Acid (sponsored by the U.S. Army Laboratories at Fort Detrick, and the AIBS), Frederick, Maryland
Curtiss, Roy, III (and D. R. Stallions)	Probability of F integration and frequency of stable Hfr donors in F^+ populations of *Escherichia coli* (Bacteriol. Proc., p. 55, 1968)	American Society for Microbiology (68th Annual Meeting), Detroit, Michigan
Curtiss, Roy, III (D. R. Stallions, L. G. Caro, and D. P. Allison)	Early stages during conjugation in *Escherichia coli* K-12	American Society for Microbiology (Joint Meeting of the Kentucky-Tennessee, Ohio, and Indiana Branches), Louisville, Kentucky
Darden, E. B., Jr.	Influence of hypoxia on late effects of ionizing radiation in mice	UT-AEC Agricultural Research Laboratory Seminar, Oak Ridge, Tennessee
Darden, E. B., Jr. (K. W. Christenberry, M. C. Jernigan, and J. W. Conklin)	Influence of hypoxia on late somatic effects of radiation in mice: Survival and cataract (Radiation Res., 35: 555, 1968)	Radiation Research Society (16th Annual Meeting), Houston, Texas
Darden, E. B., Jr. (and M. C. Jernigan)	Radiation methods in mammalian radiobiological studies (lecture and demonstration)	Public Awareness Seminars, Oak Ridge Regional Science Center, Oak Ridge, Tennessee
de Serres, F. J.	The biological effects of space flight during the Biosatellite II mission	University of Tennessee, Knoxville
	Biological effects of weightlessness on the Biosatellite II mission	University of Tennessee Medical Center, Memphis
	Dose-rate effects on the spectrum of recessive lethal mutation in the *ad-3* region of *Neurospora crassa* (Genetics, 60: 173, 1968)	(1) Genetics Society of America (37th Annual Meeting), Boston, Massachusetts (2) International Symposium on Genetic Effects of Radiation and Radiomimetic Chemicals, Kyoto, Japan
	The effect of X-irradiation under anoxia on the frequency and type of recessive lethal mutations at the *ad-3A* and *ad-3B* loci of *Neurospora crassa* (Radiation Res., 35: 524, 1968)	Radiation Research Society (16th Annual Meeting), Houston, Texas
	The effect of X-rays on inactivation and mutation-induction in *Neurospora crassa*	Donner Laboratory, University of California, Berkeley

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
de Serres, F. J.	The NASA Biosatellite radiation experiments	NASA Bio-Space Technology Training Program, Wallops Island, Virginia
	The Neurospora experiment on the Biosatellite II and Gemini XI missions	University of Tokyo, Tokyo, Japan
	Radiation and chemical mutagenesis in *Neurospora crassa*	Stanford University, Stanford, California
	Recent results of microorganism and plant experiments on United States spaceflight missions	COSPAR Meeting, Tokyo, Japan
de Serres, F. J. (and H. V. Malling)	Identification of the genetic alterations in specific locus mutants at the molecular level (In *Proceedings of the XII International Congress of Genetics, Tokyo, Japan, August 19–28, 1968; Vol. II – Abstracts of Plenary Lectures, Invited Lectures, and Small Symposia.* The Science Council of Japan, Tokyo, Japan, 1968, p. 129)	XIIth International Congress of Genetics, Tokyo, Japan
de Serres, F. J. (and B. B. Webber)	The combined effect of weightlessness and radiation on inactivation and mutation-induction in *Neurospora crassa* during the Biosatellite II mission (BioScience, **18:** 590–595, 1968)	Symposium on the Biosatellite II Experiments – Preliminary Results. Sponsored by NAS-SSB and NASA, Washington, D.C.
	Effects of weightlessness on radiation-induced inactivation and mutation-induction in *Neurospora crassa*	International Symposium on Genetic Effects of Space Environment, Tokyo, Japan
	Genetic effects of ^{85}Sr gamma irradiation on *Neurospora crassa* on the Biosatellite II mission (Radiation Res., **35:** 499–500, 1968)	Radiation Research Society (16th Annual Meeting), Houston, Texas
Doherty, D. G. (and Jesse James)	Pepsin-catalyzed hydrolysis of picolinoyl-, nicotinoyl-, and isonicotinoyl-L-phenylalanyl-L-phenylalanine ethyl ester (Federation Proc., **27:** 784, 1968)	Federation of American Societies for Experimental Biology (Am. Soc. Biol. Chem.), Atlantic City, New Jersey
Donnellan, J. E., Jr. (and R. S. Stafford)	The UV photoproducts of bacterial spores (Abstr. of 5th Intern. Congr. Photobiol., 1968, p. Bc-4)	Fifth International Congress of Photobiology, Hanover, New Hampshire
Donnellan, J. E., Jr. (R. S. Stafford, J. L. Hosszu, and R. O. Rahn)	Evidence for the lethal contribution of photoproducts in *B. megaterium* spores (Abstr. of papers, 12th Ann. Meet., Biophys. Soc., 1968, p. A-84)	Biophysical Society (12th Annual Meeting), Pittsburgh, Pennsylvania
Dumont, J. N.	The origin of cortical alveoli in *Amphioxus* oocytes (Anat. Record, **160:** 469, 1968)	American Association of Anatomists, New Orleans, Louisiana
	Some aspects of oocyte development and maturation in the annelid, *Enchytraeus albidus*	Tenth Southeastern Developmental Biology Conference, Wakulla Springs, Florida
Dumont, J. N. (and R. A. Wallace)	The synthesis, transport, and uptake of yolk proteins in *Xenopus laevis* (J. Cell Biol., **39:** 37a, 1968)	American Society for Cell Biology, Boston, Massachusetts
Eckhardt, R. A.	Observations on cyclic patterns of RNA synthesis in *Rhynchosciara* (J. Cell Biol., **39:** 39a, 1968)	American Society for Cell Biology, Boston, Massachusetts
	Observations on oogenesis in *Rhynchosciara*	AIBS, Columbus, Ohio
Ehling, U. H. (D. G. Doherty, and H. V. Malling)	Differential mutagenic action of n- and iso-propyl methanesulfonate in mice (In *Proceedings of the XII International Congress of Genetics, Tokyo, Japan, August 19–28, 1968; Vol. I – Abstracts of Contributed Papers.* The Science Council of Japan, Tokyo, Japan, 1968, p. 103)	XIIth International Congress of Genetics, Tokyo, Japan

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Einstein, J. R.	An analytical method for single-crystal equatorial-plane diffractometry	Georgia Institute of Technology, Atlanta
Einstein, J. R. (C. H. Wei and J. R. Einstein)	Molecular structure of thymine (*cis-syn*) photodimer	American Crystallographic Association, Buffalo, New York
Epler, J. L.	(1) Protein synthesis (2) Fractionation of transfer RNA by column chromatography (3) Laboratory methods in the isolation of nucleic acids	Course on Macromolecular Separations, Institute for Scientific Investigation, Caracas, Venezuela
	Mitochondrial-specific transfer RNA's and synthetases	(1) Georgetown University, Washington, D.C. (2) North Carolina A&T State University, Greensboro
	Transfer of genetic information	U.S. Naval Reserve Seminar, Oak Ridge, Tennessee
	Mitochondrial RNAs	Central University, Caracas, Venezuela
Epler, J. L. (and W. E. Barnett)	The cytoplasmic and mitochondrial tRNA's of Neurospora – Fractionation, coding properties, and interspecific reactions	Florida State University Alumni Studies Conference on Molecular Biology, Tallahassee
Ewing, R. D.	A new cytidine diphosphate compound in *Artemia salina*	American Chemical Society (Southeastern Regional Meeting), Tallahassee, Florida
Finamore, F. J.	Control of DNA synthesis by unusual nucleotides during development and differentiation (3 lectures)	Texas A&M University, College Station
	Role of nucleotides in the differentiation of the brine shrimp, *Artemia salina*	American Chemical Society (Southeastern Regional Meeting), Tallahassee, Florida
Fisher, W. D.	Characterization and isolation of macromolecules and cell constituents by centrifugal techniques	Course on Macromolecular Separations, Institute for Scientific Investigation, Caracas, Venezuela
Fisher, W. D. (H. I. Adler, and F. W. Shull, Jr.)	Induction of cell division in bacterial filaments by cell extracts (J. Cell Biol., 39: 45a, 1968)	American Society for Cell Biology, Boston, Massachusetts
Foard, D. E.	Pattern of incorporation of labeled nucleic acid precursors in wheat root pericycle (ASB Bull., 15: 37, 1968)	Association of Southeastern Biologists (29th Annual Meeting) (Southern Section, Am. Soc. Plant Physiol.), Athens, Georgia
Fralick, J. A. (W. D. Fisher, and H. I. Adler)	Poly-U directed poly ϕ alanine synthesis in minicell extracts	American Society for Microbiology (Joint Meeting of the Kentucky-Tennessee, Ohio, and Indiana Branches), Louisville, Kentucky
Fujimura, R. K.	Biochemical analysis of the recombination-mechanism of DNA strand of T5 bacteriophage	Oak Ridge Associated Universities, Oak Ridge, Tennessee
Fujimura, R. K. (and Elliot Volkin)	Analysis of naturally, covalently rejoined section of DNA strands (Federation Proc., 27: 396, 1968)	Federation of American Societies for Experimental Biology (Am. Soc. Biol. Chem.), Atlantic City, New Jersey
Fuller, R. C.	Carbon dioxide fixation in green photosynthetic bacteria	American Society for Microbiology (68th Annual Meeting), Detroit, Michigan
	The comparative biochemistry of CO_2 fixation	International Conference on Photosynthesis in Sugar Cane, London, England

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Fuller, R. C.	The comparative biochemistry of CO_2 fixation in photosynthetic bacteria	First International Congress of Photosynthesis, Freudenstadt, Germany
	Light and life	Sigma Xi, University of Tennessee Chapter, Knoxville
	Some aspects of structure and function in bacterial photosynthesis	Amherst College, Amherst, Massachusetts
	Some characteristics of enzymes and the control of bacterial photosynthesis	University of Massachusetts, Amherst
Gaulden, Mary Esther	Prevention of X-ray-induced mitotic inhibition by post-irradiation exposure of cells to hypertonic culture medium (Radiation Res., **35**: 523, 1968)	Radiation Research Society (16th Annual Meeting), Houston, Texas
Generoso, W. M.	Chemical induction of dominant lethals in female mice (Genetics, **60**: 181–182, 1968)	Genetics Society of America (37th Annual Meeting), Boston, Massachusetts
Gengozian, Nazareth (and C. C Congdon)	Effect of syngeneic antilymphocyte serum on rat-mouse radiation chimeras	Second International Congress of the Transplantation Society, New York City
Goodman, Joan Wright	Factors that influence the growth of transplanted hemopoietic cells	UT-AEC Agricultural Research Laboratory Seminar, Oak Ridge, Tennessee
Goodman, Joan Wright (and Sarah G. Shinpock)	Ability of thymus cells to improve poor growth of transplanted bone marrow (Federation Proc., **27**: 506, 1968)	Federation of American Societies for Experimental Biology (Am. Assoc. Immunol.), Atlantic City, New Jersey
Goodman, Joan Wright (Sarah G. Shinpock, and H. B. Wheeler)	An apparently nonimmunologic effect of thymus cells	2nd International Congress of the Transplantation Society, New York City
Grell, E. H.	Genetic alterations of alpha-glycerophosphate dehydrogenase in *Drosophila melanogaster* (Genetics, **60**: 184, 1968)	Genetics Society of America (37th Annual Meeting), Boston, Massachusetts
	Some aspects of genetic control of enzymes in *Drosophila*	Princeton University, Princeton, New Jersey
Grell, Rhoda F.	The distributive model and w^{m4} (Genetics, **60**: 184, 1968)	Genetics Society of America (37th Annual Meeting), Boston, Massachusetts
	Meiosis – application of basic knowledge to humans	Symposium on Human Cytogenetics, University of Tennessee Memorial Research Center and Hospital, Knoxville
	Meiosis – application of basic principles to human cytogenetics	Sigma Xi Lecture, George Washington University, Washington, D.C.
	Meiotic chromosome behavior (2 lectures)	Case Western Reserve University, Cleveland, Ohio
Groves, D. L.	Assessment of hemolytic plaque-forming cells (PFC) in Millipore diffusion chambers (Federation Proc., **27**: 318, 1968)	Federation of American Societies for Experimental Biology (Am. Assoc. Immunol.), Atlantic City, New Jersey
	Interaction of cell types in the generation of antibody-forming cells – a model (RES: J. Reticuloendothelial Soc., **5**: 561, 1968)	Reticuloendothelial Society (5th National Meeting), New York City
Gude, W. D.	Autoradiography (2 lectures, 1 laboratory)	Course on Macromolecular Separations, Institute for Scientific Investigation, Caracas, Venezuela

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Gude, W. D.	(1) Biological effects of radiation on mammals (2) Careers in laboratory technology	(1) Powell High School, Powell, Tennessee (2) Doyle High School, Knoxville, Tennessee (3) Brainerd High School, Chattanooga, Tennessee
Haber, A. H.	Development and senescence: Two sides of the same coin	Washington University, St. Louis, Missouri
	Plant morphogenesis and common sense	(1) University of Wisconsin, Milwaukee (2) Tulane University, New Orleans, Louisiana
	Plant morphogenesis against common sense	Purdue University, Lafayette, Indiana
Haber, A. H. (and D. E. Foard)	Effects of seedling irradiation on growth of gamma plantlets [Plant Physiol., **43** (Suppl.): S-49, 1968]	American Society of Plant Physiologists, Amherst, Massachusetts
Haber, A. H. (Paula J. Thompson, Patricia L. Walne, and L. L. Triplett)	Light-retardation of functional and ultrastructural chloroplast deterioration independent of photosynthesis (Abstr. of 5th Intern. Congr. Photobiol., 1968, p. Ee-1)	Fifth International Congress of Photobiology, Hanover, New Hampshire
Haber, A. H. (and L. L. Triplett)	Cancelling errors in the quantitative determination of α-amylase activity (ASB Bull., **15**: 38, 1968)	Association of Southeastern Biologists (29th Annual Meeting) (Southern Section, Am. Soc. Plant Physiol.), Athens, Georgia
Hager, C. B.	Hormonal control of tyrosine transaminase in the isolated, perfused rat liver (Federation Proc., **27**: 641, 1968)	Federation of American Societies for Experimental Biology (Am. Soc. Biol. Chem.), Atlantic City, New Jersey
Hamkalo, Barbara A. (and P. A. Swenson)	ATP levels in irradiated *Escherichia coli* B/r (Abstr. of 5th Intern. Congr. Photobiol., 1968, p. Gh-5)	Fifth International Congress of Photobiology, Hanover, New Hampshire
	Physiological changes in UV-irradiated *Escherichia coli* (Abstr. of papers, 12th Ann. Meet., Biophys. Soc., 1968, p. A-85)	Biophysical Society (12th Annual Meeting), Pittsburgh, Pennsylvania
Hanna, M. G., Jr.	Studies of antigen localization in spleen lymphatic germinal centers	Oak Ridge Associated Universities, Oak Ridge, Tennessee
Hanna, M. G., Jr. (Mary W. Francis, and Leona C. Peters)	Localization of ^{125}I-labeled antigen in germinal centers of mouse spleen: Effects of competitive injection of specific or noncrossreacting antigen (Exptl. Hematol., **17**: 15–16, 1968)	Second International Conference on Germinal Centers of Lymphatic Tissue, Padova, Italy
Hanna, M. G., Jr. (Paul Nettesheim, and H. E. Walburg, Jr.)	A comparative study of the immune reaction in germ-free and conventional mice	Symposium on Gnotobiology, Roswell Park, Buffalo, New York
Hanna, M. G., Jr. (A. K. Szakal, Paul Nettesheim, and H. E. Walburg, Jr.)	The relation of antigen localization to the development and growth of lymphoid germinal centers	Second International Conference on Germinal Centers of Lymphatic Tissue, Padova, Italy
Hardigree, Alice A. (H. I. Adler and Alice A. Hardigree)	Growth and division of individual cells of *Escherichia coli* observed with time lapse cinematographic techniques	American Society for Microbiology (Joint Meeting of the Kentucky-Tennessee, Ohio, and Indiana Branches), Louisville, Kentucky
Harrison, A. P., Jr.	Studies on *in vivo* protein photooxidation by monitoring tritium loss from *in vivo* histidine moieties (Abstr. of 5th Intern. Congr. Photobiol., 1968, p. Be-4)	Fifth International Congress of Photobiology, Hanover, New Hampshire

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Hartman, F. C.	A potential active site reagent for aldolase, triose phosphate isomerase, and glycerol-1-phosphate dehydrogenase (Federation Proc., 27: 454, 1968)	Federation of American Societies for Experimental Biology (Am. Soc. Biol. Chem.), Atlantic City, New Jersey
Hilse, K. M.	Linked genes as the basis of amino acid duplexity in α-chains of mouse Hbs	(1) University of Tennessee Medical School, Memphis (2) California Institute of Technology, Pasadena
Hollaender, Alexander	Introductory and closing remarks	International Symposium on Nuclear Physiology and Differentiation, Belo Horizonte, Brazil
	Radiation protection and recovery from a biophysical and genetical point of view	The German Biophysical Society, Berlin, Germany
Hollaender, Alexander (and D. G. Doherty)	The background for modification of radiation damage by sulfur compounds	Panel Meeting on The Radiation Damage to the Biological Molecular Information System with Special Regard to the Role of SH-Groups, IAEA, Vienna, Austria
Hooper, Dona C. (and J. F. Albright)	The influence of environmental factors on the growth of myeloma plasma cells (Federation Proc., 27: 688, 1968)	Federation of American Societies for Experimental Biology (Am. Assoc. Immunol.), Atlantic City, New Jersey
Jacobson, K. Bruce (J. A. Knopp, and J. B. Murphy)	Alcohol dehydrogenase from *Drosophila:* Interconversion of isoenzymes (Federation Proc., 27: 590, 1968)	Federation of American Societies for Experimental Biology (Am. Soc. Biol. Chem.), Atlantic City, New Jersey
	Properties of the isoenzymes of *Drosophila* alcohol dehydrogenase	Isozyme Conference, Galveston, Texas
Jenkins, V. K.	Alteration of splenic hemopoiesis in RF mice by estradiol treatment	Oklahoma Medical Research Foundation, Oklahoma City
	Spleen colony formation in RF mice – effect of estradiol treatment	Baylor Medical School, Houston, Texas
Jenkins, V. K. (A. C. Upton, and T. T. Odell, Jr.)	Estradiol-induced alteration of hemopoiesis in RFM mice (Federation Proc., 27: 723, 1968)	Federation of American Societies for Experimental Biology (Am. Soc. Exptl. Pathol.), Atlantic City, New Jersey
Jolley, R. L. (and H. V. Malling)	Man or mouse? A comparison of urinary carbohydrate constituents using high resolution column chromatography	American Chemical Society (Southeastern Regional Meeting), Tallahassee, Florida
Kelmers, A. D. (R. L. Pearson, L. R. Shugart, and J. F. Weiss)	Preparation of two formylmethionine transfer RNAs from *E. coli* B by a new reversed-phase chromatographic method (Federation Proc., 27: 342, 1968)	Federation of American Societies for Experimental Biology (Am. Soc. Biol. Chem.), Atlantic City, New Jersey
Kenney, F. T.	Control of enzyme synthesis and turnover	American Chemical Society (Southeastern Regional Meeting), Tallahassee, Florida
	Hormonal induction and repression	Third Kettering Symposium: Some Regulatory Mechanisms for Protein Synthesis in Mammalian Cells, Yellow Springs, Ohio
	Hormonal regulation of enzyme synthesis	University of Windsor, Windsor, Ontario, Canada

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Kenney, F. T.	Regulation of enzyme synthesis and turnover	Case Western Reserve University, Cleveland, Ohio
	Regulation of enzyme synthesis and turnover in mammalian tissues	University of Connecticut, Storrs
Kenney, F. T. (and J. R. Reel)	Hormonal regulation of enzyme synthesis: Transcriptional or translational control?	Conference on Hormones in Development, Nottingham, England
Kerr, Marilyn S.	Evidence for extra-oocytic origin of yolk protein in the blue crab, *Callinectes sapidus*	Tenth Southeastern Developmental Biology Conference, Wakulla Springs, Florida
	Protein synthesis by hemocytes of *Callinectes sapidus* (J. Cell Biol., 39: 72a, 1968)	American Society for Cell Biology, Boston, Massachusetts
Khym, J. X.	The change in distribution coefficients of purines and pyrimidines by pretreatment of Sephadex columns	American Chemical Society (Southeastern Regional Meeting), Tallahassee, Florida
	Determination of sequence in polyribonucleotides by the action of amines on periodate-oxidized nucleotide residues (Abstr. of papers, 155th Meet., Am. Chem. Soc., 1968, C-41)	Symposium on Sugar Phosphates and Sugar Nucleotides, American Chemical Society (155th National Meeting), San Francisco, California
Kimball, R. F.	Cellular recovery and repair	ORAU Medical Division Seminar, Oak Ridge, Tennessee
	The effect of repair on mutation induction by radiation and by chemicals	(1) Paterson Laboratories, Manchester, England (2) University of London, London, England
	Induction of mutations in Paramecium by ultraviolet and X rays in relation to repair processes	Conference of the Photobiology Group and Radiobiology Section of the British Institute of Radiology, London, England
	Opening remarks	First Southeastern Immunology Workshop, Oak Ridge, Tennessee
	Repair in mutation process. Microorganism level (In *Proceedings of the XIIth International Congress of Genetics, Tokyo, Japan, August 19–28, 1968; Vol. II – Abstracts of Plenary Lectures, Invited Lectures, and Small Symposia.* The Science Council of Japan, Tokyo, Japan, 1968, p. 136)	XIIth International Congress of Genetics, Tokyo, Japan
	Research programs at the Biology Division, ORNL	ORAU East Central College Consortium, Oak Ridge, Tennessee
Knopp, J. A. (K. Bruce Jacobson, Peter Pfuderer, and J. W. Longworth)	Alcohol dehydrogenase from Drosophila: Characterization and interconversion of isoenzymes (Abstr. of papers, 156th Meet., Am. Chem. Soc., 1968, Biol. No. 178)	American Chemical Society (156th National Meeting), Atlantic City, New Jersey
Kretchmar, A. L.	Early proliferation of transplanted spleen (Exptl. Hematol., 16: 14, 1968)	Bone Marrow Conference, Atlantic City, New Jersey
Kull, F. J. (and K. Bruce Jacobson)	Multiple phenylalanine-tRNA synthetases in *Neurospora crassa* (Abstr. of papers, 156th Meet., Am. Chem. Soc., 1968, Biol. No. 238)	American Chemical Society (156th National Meeting), Atlantic City, New Jersey
Leibo, S. P. (John Farrant, Peter Mazur, M. G. Hanna, Jr., and L. H. Smith)	Freezing of mouse marrow stem cells: Interactions of cooling and warming rates in the presence of PVP, sucrose, or glycerol	Bone Marrow Conference, New York City
Leibo, S. P. (and Peter Mazur)	Freezing of bacteriophage T4Bos (Cryobiology, 4: 252, 1968)	Society for Cryobiology (5th Annual Meeting), Washington, D.C.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Longworth, J. W.	Techniques for measuring phosphorescence and fluorescence of biological molecules (Abstr. of 5th Intern. Congr. Photobiol., 1968, p. Ka-4)	Fifth International Congress of Photobiology, Hanover, New Hampshire
Longworth, J. W. (J. A. Knopp, J. J. ten Bosch, and R. O. Rahn)	Electronic energy transfer in oligomers and polymers of L-tyrosine	International Conference on Molecular Luminescence, Chicago, Illinois
Makinodan, Takashi	Aging and immune competence	Institute for Scientific Investigation, Caracas, Venezuela
	Cellular aspects of antibody response	University of Illinois, Urbana
	Cellular aspects of immune response	Institute for Scientific Investigation, Caracas, Venezuela
	Cytokinetics of antibody response	University of Illinois Medical Center, Chicago
	Cytokinetics of the immune response (series of 3 lectures)	University of Puerto Rico, San Juan
	Immune competence and aging	(1) Institute for Scientific Investigation, Caracas, Venezuela (2) ORAU Medical Division, Oak Ridge, Tennessee
	Immunological aspects of aging	(1) Research Committee Meeting, ORNL, Oak Ridge (2) Ortho Research Foundation, Raritan, New Jersey (3) Reactor Division Seminar, ORNL, Oak Ridge
	Immunological significance of antigen-induced proliferation *in vivo*	Federation of American Societies for Experimental Biology (Symposium on the Role of Proliferative Events in the Development of Immune Responses), Atlantic City, New Jersey
	In vivo and *in vitro* studies of factors effecting enhancement or suppression of immune response	Midwinter Conference of Immunologists, Houston, Texas
	Mechanism of antibody response	(1) Veterans Administration Hospital, Washington, D.C. (2) University of Georgia, Athens
Malling, H. V. (and F. J. de Serres)	Genetic analysis of hydroxylamine-induced purple mutants (*ad-3*) in *Neurospora crassa* (In *Proceedings of the XII International Congress of Genetics, Tokyo, Japan, August 19–28, 1968; Vol. I – Abstracts of Contributed Papers.* The Science Council of Japan, Tokyo, Japan, 1968, p. 83)	XIIth International Congress of Genetics, Tokyo, Japan
	Identification of the spectrum of X-ray-induced intragenic alterations at the molecular level in *Neurospora crassa*	International Symposium on Genetic Effects of Radiation and Radiomimetic Chemicals, Kyoto, Japan
	Mutagenicity of alkylating carcinogens	Conference on Biological Effects of Alkylating Agents, New York City
	The relationship between carcinogenicity and mutagenicity of methylating compounds (Genetics, **60**: 201, 1968)	Genetics Society of America (37th Annual Meeting), Boston, Massachusetts
Malling, H. V. (U. H. Ehling and H. V. Malling)	1,4-di(methane-sulfonoxy) butane (Myleran) as a mutagenic agent in mice (Genetics, **60**: 174–175, 1968)	Genetics Society of America (37th Annual Meeting), Boston, Massachusetts

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Mattingly, Ellen	Observations on the limited chromosome in *Rhynchosciara angelae* (J. Cell Biol., **39**: 87a, 1968)	American Society for Cell Biology, Boston, Massachusetts
	Polytene chromosomes: A useful tool for cytologists	Spring Hill College, Mobile, Alabama
	Recent studies on chromosome metabolism in *Rhynchosciara*	University of North Carolina, Greensboro
	Studies on nucleic acid synthesis in *Rhynchosciara* (2 lectures)	University of Tennessee, Knoxville
Mazur, A. (and L. H. Smith)	Ferrochelatase activity as an index of erythroid cell activity in tissues of spleen-shielded rats recovering from X-irradiation	Bone Marrow Conference, New York City
Mazur, Peter	The freezing of living cells	University of Notre Dame, South Bend, Indiana
	Physico-chemical factors involved in freezing injury	Army Institute of Environmental Medicine, Natick, Massachusetts
	The response of cells and tissues to freezing and thawing at various rates	The Retina Society, Boston, Massachusetts
	Responses of cells to freezing	Georgia Institute of Technology, Atlanta, Georgia
Mazur, Peter (John Farrant, S. P. Leibo, and E. H. Y. Chu)	Freezing and thawing of Chinese hamster cells: Interactions between the optimum cooling velocity for survival and the nature and concentration of protective additive	Bone Marrow Conference, New York City
McArthur, W. H.	Morphologic basis for liver enlargement (Exptl. Hematol., **16**: 2–3, 1968)	Bone Marrow Conference, Atlantic City, New Jersey
McDevitt, D. S.	Ontogeny of lens-specific proteins in embryonic and larval (metamorphic) *Rana pipiens* (J. Cell Biol., **39**: 88a–89a, 1968)	American Society for Cell Biology, Boston, Massachusetts
McDonald, T. P. (R. D. Lange, C. C Congdon, M. L. Simmons, and R. E. Toya)	Effect of hypoxia, irradiation, and bone marrow transplantation on erythropoietin production in mice	XIIth Congress of the International Society of Hematology, New York City
Miller, O. L., Jr.	Fine structure of genetic redundancy in the nucleolus	(1) Institute of Scientific Investigation, Caracas, Venezuela (2) University of the Rio Grande of the South, Porto Alegre, Brazil (3) Institute of General Anatomy and Embryology, Buenos Aires, Argentina (4) University of La Plata, La Plata, Argentina
	Nucleolar structure and function	University of Tennessee, Knoxville
Miller, O. L., Jr. (and Barbara R. Beatty)	Amphibian oocyte nucleoli – structure and function (J. Cell Biol., **39**: 156a, 1968)	American Society for Cell Biology, Boston, Massachusetts
	Extrachromosomal nucleolar genes in amphibian oocytes	International Symposium on Nuclear Physiology and Differentiation, Belo Horizonte, Brazil
Mitchell, T. J. (C. C Congdon, M. A. Kastenbaum, and D. A. Gardiner)	Factorial design–response surface study of mortality from secondary disease in mouse radiation chimeras (Federation Proc., **27**: 307, 1968)	Federation of American Societies for Experimental Biology (Am. Soc. Exptl. Pathol.), Atlantic City, New Jersey
Modak, S. P.	DNA synthesis and DNA breakdown during lens cell differentiation	University of Pennsylvania, Philadelphia

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Modak, S. P. (and Stella W. Perdue)	Studies on nuclear degeneration during the terminal lens differentiation (J. Cell Biol., **39:** 173a, 1968)	American Society for Cell Biology, Boston, Massachusetts
Modak, S. P. (R. C. von Borstel, and F. J. Bollum)	A study of nuclear degeneration using calf thymus DNA polymerase (J. Cell Biol., **39:** 93a, 1968)	American Society for Cell Biology, Boston, Massachusetts
Montour, J. L. (E. B. Darden, Jr., and A. C. Upton)	Acute effects of 62 MeV protons in mice (Radiation Res., **35:** 576–577, 1968)	Radiation Research Society (16th Annual Meeting), Houston, Texas
Moseley, B. E. B.	The molecular basis of repair in *Micrococcus radiodurans*	Chalk River, Ontario, Canada
	Repair of UV damage in sensitive mutants of *Micrococcus radiodurans* (Abstr. of papers, 12th Ann. Meet., Biophys. Soc., 1968, p. A-55)	Biophysical Society (12th Annual Meeting), Pittsburgh, Pennsylvania
	Repair of UV damage in wild-type and radiation-sensitive mutants of *Micrococcus radiodurans* (Abstr. of 5th Intern. Congr. Photobiol., 1968, p. Hf-1)	Fifth International Congress of Photobiology, Hanover, New Hampshire
Nettesheim, Paul (and M. G. Hanna, Jr.)	Radiosensitivity of the antigen trapping mechanism and its relation to the suppression of the immune response (Exptl. Hematol., **17:** 16, 1968)	Second International Conference on Germinal Centers of Lymphatic Tissue, Padova, Italy
Nöthiger, Rolf	Changes in cellular structure and synthetic activities during lens formation	Brandeis University, Waltham, Massachusetts
Novelli, G. David	(1) Amino acid activation for protein synthesis (2) Changes in isoaccepting tRNA's during different metabolic states	Temple University School of Medicine, Philadelphia, Pennsylvania
	New chromatographic systems for the separation of individual species of transfer RNA and their analytical use	McMaster University, Hamilton, Ontario, Canada
	New systems for isolation of individual species of isoaccepting tRNAs and their analytical use	International Symposium on Molecular Biology, New York City
	New systems for the separation of individual species of tRNA and their analytical use	(1) American Society for Microbiology (Joint Meeting of the Kentucky-Tennessee, Ohio, and Indiana Branches), Louisville, Kentucky (2) American Society for Microbiology (Central New York Branch), Buffalo, New York
	Quantity production of transfer RNA	U.S. Naval Reserve Seminar, Oak Ridge, Tennessee
	Role of tRNA in the regulation of protein synthesis	Third Kettering Symposium: Some Regulatory Mechanisms for Protein Synthesis in Mammalian Cells, Yellow Springs, Ohio
	Studies on transfer RNA	(1) University of Pennsylvania School of Medicine, Philadelphia (2) St. Louis University School of Medicine, St. Louis, Missouri (3) University of Georgia, Athens (4) Rutgers University, New Brunswick, New Jersey (5) Columbia University, New York City (6) Los Alamos Scientific Laboratory, Los Alamos, New Mexico

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Nugent, N. A. (and R. C. Fuller)	Pteridine as a cofactor in carotenoid desaturation in *Rhodospirillum rubrum* [Plant Physiol., **43** (Suppl.): S-13, 1968]	American Society of Plant Physiologists, Amherst, Massachusetts
Oakberg, E. F.	Mammalian gametogenesis and species comparisons in radiation response of the gonads (In *Effects of Radiation on Meiotic Systems, Report of a Study Group on the Effects of Radiation on Meiotic Systems.* Organized by the International Atomic Energy Agency, Vienna, Austria, 1968, pp. 3–15)	Conference on The Effects of Ionizing Radiation on Meiotic Systems, Vienna, Austria
	Radiation effects on the gonads	University of Tennessee College of Medicine, Memphis
	Radiation effects on spermatogenesis	Third International Congress of Endocrinology, Mexico City, Mexico
O'Dell, D. H. (and D. E. Foard)	Using colchicine to determine the number of cells initially forming a lateral root primordium (ASB Bull., **15**: 48, 1968)	American Society of Plant Physiologists (Southern Section), Athens, Georgia
Odell, T. T., Jr.	Steady state renewal of cells – the megakaryocyte-platelet system	Public Awareness Seminars, Oak Ridge Regional Science Center, Oak Ridge, Tennessee
Odell, T. T., Jr. (C. W. Jackson, and Rebecca S. Reiter)	Generation cycle of rat megakaryocytes (Federation Proc. **27**: 672, 1968)	Federation of American Societies for Experimental Biology (Am. Soc. Exptl. Pathol.), Atlantic City, New Jersey
Odell, T. T., Jr. (C. W. Jackson, Rebecca S. Reiter, and Deborah E. Charsha)	Studies on megakaryocyte regulation	XIIIth Congress of the International Society of Hematology, New York City
Ortwerth, B. J. (Ugo Del Monte, Lawrence Rosen, and G. David Novelli)	Changes in rat liver tRNA during ethionine feeding (Federation Proc., **27**: 803, 1968)	Federation of American Societies for Experimental Biology (Am. Soc. Biol. Chem.), Atlantic City, New Jersey
Pal, B. C. (Mayo Uziel, D. G. Doherty, and W. E. Cohn)	The action of alkali and acid on bis(1-methyl-4-thiouracil) disulfide (Abstr. of papers, 156th Meet., Am. Chem. Soc., 1968, Biol.-213)	American Chemical Society (156th National Meeting), Atlantic City, New Jersey
Palmer, Winifred G.	α-Crystallin, a study of its structure and function	(1) Duke University Medical School, Durham, North Carolina (2) University of New Hampshire, Durham
	Possible controls of α-crystallin synthesis	Symposium on Radiation Biology of the Ocular Lens, Rochester, Michigan
Papaconstantinou, John	Protein and nucleic acid synthesis in development (5 lectures)	Melbourne High School, Melbourne, Florida
	Regulation of RNA synthesis during lens cell differentiation	(1) University of Pennsylvania, Philadelphia (2) University of Georgia, Athens
Papaconstantinou, John (and Emilia M. Julku)	The regulation of ribosomal RNA synthesis and ribosomal assembly in lens cell differentiation	International Symposium on the Biochemistry of the Eye, Nijmegen, Holland
	The regulation of ribosomal RNA synthesis and ribosomal assembly in the vertebrate lens	Symposium on Molecular Aspects of Differentiation, Gatlinburg, Tennessee (21st Annual ORNL Biology Division Research Conference)

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Pearlstein, R. M.	Coherent versus incoherent excitons in biomolecular systems	University of Rochester, Rochester, New York
	Donor fluorescence as a probe of energy transfer	Second International Conference on Photosensitization in Solids, Tucson, Arizona
	Fast coincidence device for subnanosecond resolution of beta-particle-excited fluorescence profiles (Abstr. of 5th Intern. Congr. Photobiol., 1968, p. Dh-1)	Fifth International Congress of Photobiology, Hanover, New Hampshire
Perkins, E. H. (Toshihiko Sado, and Takashi Makinodan)	Cellular kinetics of primary antibody response (RES: J. Reticuloendothelial Soc., **5**: 560–561, 1968)	Reticuloendothelial Society (5th National Meeting), New York City
Pfuderer, Peter	The amino acid incorporating activity of isolated polysomes (Federation Proc., **27**: 803, 1968)	Federation of American Societies for Experimental Biology (Am. Soc. Biol. Chem.), Atlantic City, New Jersey
Popp, R. A.	Gene duplication as a basis for multiple alpha chains of the hemoglobin of the mouse	UT Memorial Research Center and Hospital, Knoxville, Tennessee
	(1) Mouse esterases (2) Mouse hemoglobin	University of Toledo, Toledo, Ohio
Popp, R. A. (and K. M. Hilse)	Alpha chains of hemoglobins among laboratory mice (Genetics, **60**: 212, 1968)	Genetics Society of America (37th Annual Meeting), Boston, Massachusetts
Price, G. B.	Buoyant density distributions of immunocompetent cells (RES: J. Reticuloendothelial Soc., **5**: 561–562, 1968)	(1) Reticuloendothelial Society (5th National Meeting), New York City (2) Society for Experimental Biology and Medicine (Southeastern Section), Oak Ridge, Tennessee
Price, G. B. (M. E. Boling, S. P. Modak, G. B. Price, Alice A. Mattingly, M. P. Gordon, and Jane K. Setlow)	Repair of UV damage in UV-sensitive and wild type *Hemophilus influenzae* (Abstr. of papers, 12th Ann. Meet., Biophys. Soc., 1968, p. A-54)	Biophysical Society (12th Annual Meeting), Pittsburgh, Pennsylvania
Rahn, R. O. (and J. L. Hosszu)	Factors influencing the photochemistry of DNA (Abstr. of 5th Intern. Congr. Photobiol., p. Gd-4, 1968)	Fifth International Congress of Photobiology, Hanover, New Hampshire
Rahn, R. O. (Jane K. Setlow, and J. L. Hosszu)	Temperature dependence of thymine photoproduct formation in DNA (Abstr. of papers, 12th Ann. Meet., Biophys. Soc., p. A-53, 1968)	Biophysical Society (12th Annual Meeting), Pittsburgh, Pennsylvania
Randolph, M. L.	Quantitative considerations in ESR studies of biological materials	Symposium on Tissue-ESR, Walter Reed Institute for Medical Research, Washington, D.C.
	Radiation effects on DNA	ORAU Radiation Biology Summer Institute, Oak Ridge, Tennessee
	Specific gamma-ray emission coefficient for ^{137}Cs (Radiation Res., **35**: 482, 1968)	Radiation Research Society (16th Annual Meeting), Houston, Texas
Reel, J. R.	Regulation of enzyme synthesis by hydrocortisone in hepatoma cell cultures	Third International Congress of Endocrinology, Mexico City, Mexico
Reel, J. R. (and F. T. Kenney)	Regulation of tyrosine transaminase synthesis by hydrocortisone in hepatoma cell cultures (Federation Proc., **27**: 641, 1968)	Federation of American Societies for Experimental Biology (Am. Soc. Biol. Chem.), Atlantic City, New Jersey

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Regan, James D.	Chromosomal alterations in marine fish cells *in vitro*	Sandy Hook Marine Laboratory, Sandy Hook, New Jersey
	The repair of ultraviolet-induced DNA lesions in cell cultures	Florida State University Alumni Studies Conference on Molecular Biology, Tallahassee
Regan, James D. (and J. S. Cook)	Photoreactivation among the classes of vertebrates (Abstr. of 5th Intern. Congr. Photobiol., 1968, p. Bi-6)	Fifth International Congress of Photobiology, Hanover, New Hampshire
Regan, James D. (J. S. Cook, and Susumu Takeda)	Reptilian cells *in vitro* exhibit photoreactivation	Tissue Culture Association (19th Annual Meeting). San Juan, Puerto Rico
Regan, James D. (and Susumu Takeda)	Aspects of photoreactivation in animal cell cultures (Radiation Res., **35**: 517–518, 1968)	Radiation Research Society (16th Annual Meeting), Houston, Texas
Richter, C. B.	Filter cap studies	Meeting of Laboratory Animal Breeders Association, Washington, D.C.
	Patterns of fatal enteritis in caged cottontails	American Association for Laboratory Animal Science, Las Vegas, Nevada
Richter, C. B. (and R. C. Brown)	The pathology and epidemiology of acute enteritis in cottontails, with reference to ultrastructure observations	Wildlife Disease Association (AIBS), Madison, Wisconsin
Riggsby, W. S.	Fractionation of nucleic acids by means of DNA-nitrocellulose chromatography	(1) Vanderbilt University, Nashville, Tennessee (2) Marquette University, Milwaukee, Wisconsin
	Fractionation of nucleic acids on the basis of genetic specificity	Southwest Center for Advanced Studies, Dallas, Texas
	Fractionation of nucleic acids on the basis of sequence homology	Kansas State University, Manhattan
	Nucleic acid separation on nitrocellulose columns (Federation Proc., **27**: 804, 1968)	Federation of American Societies for Experimental Biology (Am. Soc. Biol. Chem.), Atlantic City, New Jersey
	Studies with purified bacteriophage-specific messenger ribonucleic acid	University of Tennessee, Knoxville
Robie, D. M. (and H. E. Walburg, Jr.)	Ethylene-oxide sterilization of plastic-film isolators: A six-year study of operational efficiency	Association for Gnotobiotics, Buffalo, New York
Rogers, Stanfield	Concerning the transmission of genetic information with viruses	Francis Delafield Hospital, Columbia University, New York City
	The systemic effect of Shope virus induced arginase (Proc. Am. Assoc. Cancer Res., 9: 61, 1968)	American Association for Cancer Research, Atlantic City, New Jersey
	Use of viruses to transmit genetic information	(1) Conference on Molecular Biology and Pathology, Saratoga Springs, New York (2) Indiana University School of Medicine, Indianapolis
Rosen, Lawrence	2′-*O*-ethylation of rat-liver tRNA by ethionine	American Chemical Society (Southeastern Regional Meeting), Tallahassee, Florida

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Russell, Liane B.	(1) Activity of the mammalian X chromosome (2) Use of sex chromosome aberrations in mutagenic studies	Case Western Reserve University, Cleveland, Ohio
	Genetical activity of the mammalian X chromosome	University of Tennessee, Knoxville
Russell, Liane B. (and Clyde S. Montgomery)	Viability and fertility of five X;1 translocations in the mouse (Genetics, **60:** 218, 1968)	Genetics Society of America (37th Annual Meeting), Boston, Massachusetts
Russell, W. L.	Evaluation of genetic effects of radiation dose rate in mice (In *Dose Rate in Mammalian Radiation Biology. The Proceedings of a Symposium on Dose Rate in Mammalian Radiation Biology,* ed. by D. G. Brown, R. G. Cragle, and T. R. Noonan. United States Atomic Energy Commission, Division of Technical Information, 1968, p. 13.1)	Symposium on Dose Rate in Mammalian Radiation Biology, Oak Ridge, Tennessee
	Radiation genetics	University of Tennessee College of Medicine, Memphis
	Factors affecting the radiation induction of mutations in the mouse	University of Wisconsin, Madison
Russell, W. L. (and Elizabeth M. Kelly)	Effect of the interval between X-irradiation and conception on mutation frequency at specific loci in female mice (Genetics, **60:** 218–219, 1968)	Genetics Society of America (37th Annual Meeting), Boston, Massachusetts
Sado, Toshihiko	Functional and ultrastructural studies of antibody-producing cells exposed to 10,000 R in Millipore diffusion chambers (RES: J. Reticuloendothelial Soc., **5:** 562, 1968)	Reticuloendothelial Society (5th National Meeting), New York City
Serrano, L. J.	Defined mice for a low-level radiation experiment	Nuclear Science Seminar, Oak Ridge, Tennessee
	Use of defined mice for studies of radiation carcinogenesis	Society for Experimental Biology and Medicine (Southeastern Section), Oak Ridge, Tennessee
Setlow, Jane K.	Photochemistry of DNA	ORAU Radiation Biology Summer Institute, Oak Ridge, Tennessee
Setlow, Jane K. (M. E. Boling, and Alice A. Mattingly)	Repair of DNA in *Hemophilus influenzae* (Abstr. of 5th Intern. Congr. Photobiol., p. Hf-6, 1968)	Fifth International Congress of Photobiology, Hanover, New Hampshire
Setlow, Jane K. (M. E. Boling, Alice A. Mattingly, and G. B. Price)	Repair of DNA in *Hemophilus influenzae*	33rd Cold Spring Harbor Symposium on DNA Replication, Repair and Recombination, Cold Spring Harbor, New York
Setlow, Jane K. (Dianne C. Brown, and M. E. Boling)	UV-sensitive mutants of *Hemophilus influenzae* (Abstr. of papers, 12th Ann. Meet., Biophys. Soc., p. A-54, 1968)	Biophysical Society (12th Annual Meeting), Pittsburgh, Pennsylvania
Setlow, R. B.	Photochemistry of DNA *in vitro* and *in vivo* (Abstr. of 5th Intern. Congr. Photobiol., p. Db-2, 1968)	Fifth International Congress of Photobiology, Hanover, New Hampshire
	Photochemistry and photobiology of DNA	Reed College, Portland, Oregon
	The repair of damage to DNA	Symposium on Modern Biology, University of Vermont, Burlington
	Repair of UV damage to DNA	(1) San Francisco Medical Center, San Francisco, California (2) University of Oregon, Eugene

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Setlow, R. B. (and W. L. Carrier)	Photoreactivation *in vivo* of the DNA of T4 phage (Abstr. of papers, 12th Ann. Meet., Biophys. Soc., p. A-53, 1968)	Biophysical Society (12th Annual Meeting), Pittsburgh, Pennsylvania
Shugart, L. R. (G. David Novelli, and M. P. Stulberg)	Restoration of aminoacylation activity of undermethylated tRNA by *in vitro* methylation (Federation Proc., 27: 342, 1968)	Federation of American Societies for Experimental Biology (Am. Soc. Biol. Chem.), Atlantic City, New Jersey
Simmons, M. L.	The hypoxia chamber and other methods of laboratory technology	Baylor Medical School, Houston, Texas
	Laboratory animal medicine and technology	University of Tennessee, Knoxville
	The laboratory mouse as a research animal	Southwest Research Center, San Antonio, Texas
Skinner, Dorothy M.	The genome for ribosomal RNA in the land crab, *Gecarcinus lateralis* (J. Cell Biol., 39: 126a, 1968)	American Society for Cell Biology, Boston, Massachusetts
	Studies on the biological role of satellite DNA's in molting and non-molting Crustacea	6th International Congress of Embryology, Paris, France
Smith, L. H.	Bone marrow stem-cell number and radiation sensitivity	Tennessee Blood Club Meeting, Dickson, Tennessee
Smith, R. H.	Unstable temperature-sensitive mutations in Habrobracon (Genetics, 60: 227, 1968)	Genetics Society of America (37th Annual Meeting), Boston, Massachusetts
Smith, R. H. (R. C. von Borstel, and D. S. Grosch)	The biological response of Habrobracon to space flight	American Association for the Advancement of Science (Am. Soc. Zool.), Dallas, Texas
Steinberg, C. M.	Phage genetics (4 lectures)	Course on Modern Topics in Biology, University of Puerto Rico, San Juan
Stewart, J. A.	Protein and nucleic acid synthesis in lens cell differentiation	(1) University of North Carolina, Chapel Hill (2) University of Michigan, Ann Arbor
	Regulation of protein synthesis in lens cell differentiation	Stanford University, Palo Alto, California
Stine, G. J.	Isolation of specific gene fragments from *Escherichia coli*	Swarthmore College, Swarthmore, Pennsylvania
Stine, G. J. (Annie S. Angel, and Roy Curtiss III)	Possible streptomycin resistance factor in *Proteus mirabilis* and its effect on recipient ability during conjugation	American Society for Microbiology (Joint Meeting of the Kentucky-Tennessee, Ohio, and Indiana Branches), Louisville, Kentucky
Swenson, P. A.	β-Galactosidase: The effect of cold treatment on photoprotection and on the release from UV catabolite repression in *Escherichia coli* B/r (Abstr. of 5th Intern. Congr. Photobiol., p. Gh-2, 1968)	Fifth International Congress of Photobiology, Hanover, New Hampshire
	Inhibition and recovery of β-galactosidase formation in UV-irradiated *Escherichia coli* B/r (Abstr. of papers, 12th Ann. Meet., Biophys. Soc., p. A-85, 1968)	Biophysical Society (12th Annual Meeting), Pittsburgh, Pennsylvania
ten Bosch, J. J.	Energy transfer in poly-L-tyrosine at 77°K (Abstr. of papers, 12th Ann. Meet., Biophys. Soc., p. A-107, 1968)	(1) Biophysical Society (12th Annual Meeting), Pittsburgh, Pennsylvania (2) University of Tennessee, Knoxville
Tennant, R. W.	*In vitro* studies on the mode of teratogenesis by a minute DNA virus	University of West Virginia, Morgantown

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Tyndall, R. L. (N. D. Bowles, J. A. Otten, and A. C. Upton)	High titers of infectious murine leukemia virus propagated *in vitro*	Tissue Culture Association (19th Annual Meeting), San Juan, Puerto Rico
Upton, A. C.	Chairman's report	Ad Hoc Subcommittee on Environmental Pathology, NAS-NRC Committee on Pathology, Washington, D.C.
	Comparative observations on radiation-induced myeloproliferative disorders in animals and man (Exptl. Hematol., **16**: 49–50, 1968)	Symposium on Myeloproliferative Disorders of Animals and Man, Richland, Washington
	(1) Delayed effects of radiation (2) Early effects of radiation	University of Pittsburgh, Pittsburgh, Pennsylvania
	Differential tissue sensitivity in radiation protection	East Tennessee Chapter, Health Physics Society, Oak Ridge, Tennessee
	Effects of radiation on biological organisms	United States Army Nuclear Science Training Session, Oak Ridge, Tennessee
	The influence of dose rate in mammalian radiation biology: Quality effects	Symposium on Dose Rate in Mammalian Radiation Biology, Oak Ridge, Tennessee
	Observations on the role of radiation in the etiology of cancer	University of Rochester, School of Medicine and Dentistry, Rochester, New York
	Observations on the role of radiation in the pathogenesis of leukemia	New York University Medical Center, Sterling Forest, New York
	Pathogenesis of cancer	American Cancer Society (Anderson County unit), Oak Ridge, Tennessee
	Radiation and cancer	Carcinogenesis and Molecular Biology Workshop, Woods Hole, Massachusetts
	Radiation carcinogenesis	State University of New York, Stony Brook
	Radiation and carcinogenesis	University of Tennessee Medical School, Memphis
	Radiation in the cause and cure of cancer	University of Tennessee Medical School, Memphis
	Radiation pathology	University of Chicago Medical School, Chicago, Illinois
	Reflections on the problem of radiation carcinogenesis	University of Cincinnati Medical Center, Cincinnati, Ohio
	The role of biological evidence in establishing radiation protection guides	Transuranium Research Laboratory Program and Safety Seminar, Oak Ridge, Tennessee
	Role of radiation in carcinogenesis	American Society for Cytology, Cleveland, Ohio
Uziel, Mayo	Fundamentals of separation processes (2 lectures, 1 laboratory)	Central University, Caracas, Venezuela
	Structure and function of $tRNA^{Phe}$ (Federation Proc., **27**: 342, 1968)	Federation of American Societies for Experimental Biology (Am. Soc. Biol. Chem.), Atlantic City, New Jersey

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Vann, D. C.	*In vivo* and *in vitro* studies of factors effecting enhancement or suppression	Conference of Immunologists, Houston, Texas
Volkin, Elliot	The discovery of messenger RNA	Physics Division Seminar, ORNL, Oak Ridge, Tennessee
	Some properties of bacteriophage T2 mRNAs	International Symposium on Nuclear Physiology and Differentiation, Belo Horizonte, Brazil
Volkin, Elliot (R. K. Fujimura and Elliot Volkin)	Biochemical evidence for the incorporation of nucleotides during the rejoining of bacteriophage DNA fragments (Science, **160:** 438, 1968)	National Academy of Sciences Meeting, Washington, D.C.
von Borstel, R. C.	Action of DNA and RNA polymerases on chromosomes	International Symposium on Nuclear Physiology and Differentiation, Belo Horizonte, Brazil
	On the origin of spontaneous mutations (In *Proceedings of the XII International Congress of Genetics, Tokyo, Japan, August 19–28, 1968; Vol. II – Abstracts of Plenary Lectures, Invited Lectures and Small Symposia.* The Science Council of Japan, Tokyo, Japan, 1968, pp. 124–125)	XIIth International Congress of Genetics, Tokyo, Japan
	Space biology	University of California, Davis
	Super-suppressors in yeast	University of California, Berkeley
von Borstel, R. C. (Dale E. Graham, Kathleen J. La Brot, and M. A. Resnick)	Mutator activity of an x-radiation-sensitive yeast (Genetics, 60: 233, 1968)	(1) Genetics Society of America (37th Annual Meeting), Boston, Massachusetts (2) Yeast Genetic Conference, Osaka, Japan
von Borstel, R. C. (and R. H. Smith)	The role of dicentric translocations in induced dominant lethality	International Symposium on Genetic Effects of Radiation and Radiomimetic Chemicals, Kyoto, Japan
von Borstel, R. C. (R. H. Smith, D. S. Grosch, Anna R. Whiting, R. L. Amy, M. B. Baird, P. D. Buchanan, Katherine T. Cain, Ruth Ann Carpenter, A. M. Clark, A. C. Hoffman, Martha S. Jones, Sohei Kondo, Margaret J. Lane, T. J. Mizianty, Mary Lou Pardue, Joan W. Reed, Diana B. Smith, Judith A. Steen, Julie T. Tindall, and L. R. Valcovic)	Mutational response of *Habrobracon* in the Biosatellite II experiment (BioScience, **18:** 598–601, 1968)	National Academy of Sciences Meeting, Washington, D.C.
von Borstel, R. C. (R. H. Smith, D. S. Grosch, Anna R. Whiting, R. L. Amy, and Sohei Kondo)	The Habrobracon experiment in the Biosatellite II spacecraft (Radiation Res., **35:** 501, 1968)	Radiation Research Society (16th Annual Meeting), Houston, Texas
von Borstel, R. C. (R. H. Smith, D. S. Grosch, Anna R. Whiting, J. V. Slater, Brenda Buckhold, and C. A. Tobias)	Experiments with Habrobracon and Tribolium on Biosatellite II	XII International Congress of Genetics, Tokyo, Japan

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
von Borstel, R. C. (R. H. Smith, Anna R. Whiting, D. S. Grosch, Luolin S. Browning, I. I. Oster, J. V. Slater, and Brenda Buckhold)	Mutational response of Drosophila, Habrobracon, and Tribolium in the Biosatellite II experiment	COSPAR Meeting, Tokyo, Japan
Walburg, H. E., Jr.	Reticular neoplasms in germ-free mice	(1) Oak Ridge Associated Universities, Oak Ridge, Tennessee (2) Association for Gnotobiotics, Buffalo, New York
Walburg, H. E., Jr. (and G. E. Cosgrove)	Influence of microbial flora and X-radiation on reticular neoplasms of RFM mice (Proc. Am. Assoc. Cancer Res., 9: 75, 1968)	American Association for Cancer Research, Atlantic City, New Jersey
	Severity of the parent-F_1 graft-versus-host reaction in irradiated germfree mice	Association for Gnotobiotics, Buffalo, New York
Walburg, H. E., Jr. (and Edna I. Mynatt)	Hemopoietic recovery in X-irradiated mice	Baylor University College of Medicine, Houston, Texas
	Studies on hemopoietic recovery of X-irradiated mice by use of ^{125}I-labeled iododeoxyuridine (Radiation Res., **35:** 488, 1968)	Radiation Research Society (16th Annual Meeting), Houston, Texas
Wallace, R. A.	The induction, synthesis, and transport of yolk proteins and their accumulation by the oocyte	(1) Notre Dame Hospital, Montreal, Canada (2) Yale University, New Haven, Connecticut
Wallace, R. A. (and J. N. Dumont)	The induced synthesis and transport of yolk proteins and their accumulation by the oocyte in *Xenopus laevis*	Symposium on Molecular Aspects of Differentiation, Gatlinburg, Tennessee (21st Annual ORNL Biology Division Research Conference)
Waters, L. C.	tRNA studies in bacteriophage-infected *E. coli*	Purdue University, Lafayette, Indiana
Webber, B. B.	Induction and analysis of *ad-3* mutants in *Neurospora crassa* with ionizing radiations	(1) University of New York, Albany (2) University of Illinois, Chicago
	The induction with ionizing radiations of *ad-3* mutations in *Neurospora crassa* and their characterization	(1) Augusta College, Augusta, Georgia (2) Southwestern at Memphis, Memphis, Tennessee
Wei, C. H. (and J. R. Einstein)	Molecular structure of thymine (*cis-syn*) photodimer	American Crystallographic Association, Buffalo, New York
Weisberg, R. A. (and M. E. Gottesman)	The integration and excision defect of phage λ dg	(1) Stanford University, Palo Alto, California (2) University of Oregon, Eugene (3) Salk Institute, San Diego, California (4) California Institute of Technology, Pasadena (5) University of Washington, Seattle (6) Phage Meeting, Cold Spring Harbor, New York
	Interaction of λ dg with the bacterial chromosome	Lysogeny Meeting, Sorrento, Italy

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Whiting, P. W.	How long will diapause larvae of *Mormoniella* live? (ASB Bull., **15:** 59, 1968)	Association of Southeastern Biologists (29th Annual Meeting) (Southern Section Am. Soc. Plant Physiol.), Athens, Georgia
Whitson, G. L.	The fate of UV induced pyrimidine dimers in *Tetrahymena*	Queens College, New York City
Whitson, G. L. (and A. A. Francis)	Ultraviolet action spectrum of survival and formation of thymine containing pyrimidine dimers in *Tetrahymena* (Abstr. of 5th Intern. Congr. Photobiol., Eh-6, 1968)	Fifth International Congress of Photobiology, Hanover, New Hampshire
Wicks, W. D.	Biochemical regulation in animal tissues (6 lectures)	University of Pernambuco, Recife, Brazil
	Control of enzyme activity during development	(1) University of Mexico, Mexico City (2) University of San Luis Potosi, San Luis Potosi, Mexico (3) University of Nuevo Leon, Monterrey, Mexico
	Role of hormones as biochemical regulators	(1) University of São Paulo, São Paulo, Brazil (2) University of Rio de Janeiro, Rio de Janeiro, Brazil
Wittliff, J. L. (Kai-Lin Lee, and F. T. Kenney)	Induction by estrogen of RNA synthesis in amphibian liver	American Chemical Society (Southeastern Regional Meeting), Tallahassee, Florida
Yamada, Tuneo	Acquisition of lens specificity by the normal lens rudiment and lens regenerate	Symposium on Radiation Biology of the Ocular Lens, Rochester, Michigan
	An approach to control of tissue specificity	University of Virginia, Charlottesville
	Correlation between cellular synthetic activities and ultrastructure in Wolffian lens regeneration	Washington University, St. Louis, Missouri
	Nucleocytoplasmic interaction in induction	Sixth International Congress of Embryology, Paris, France
	Subcellular and molecular events in lens regeneration	International Symposium on the Biochemistry of the Eye, Nijmegen, The Netherlands
Yang, W.-K.	Differences in seryl-tRNAs in mouse plasma cell tumors (Federation Proc., **27:** 687, 1968)	Federation of American Societies for Experimental Biology (Am. Soc. Immunol.), Atlantic City, New Jersey
	Some studies on the multiple isoaccepting tRNA's in mammalian cells and tissues	National Cancer Institute, Bethesda, Maryland

VISITING LECTURERS

During the period January 1–December 31, 1968, 97 seminars were given at the Biology Division by visiting scientists from other research organizations in the United States and abroad or from other Divisions of Oak Ridge National Laboratory. These speakers represented 22 states, the District of Columbia, and 15 foreign countries – Argentina, Austria, Canada, England, Germany, India, Israel, Italy, Japan, The Netherlands, Peru, Russia, Scotland, Switzerland, and Wales. In addition to the 97 guest lectures listed below, there were numerous informal seminars at which Division members spoke about their own research.

SPEAKER	DATE	AFFILIATION	SUBJECT
Peter Alexander	5/2/68	Chester Beatty Research Institute Institute of Cancer Research Royal Cancer Hospital Belmont, Sutton, Surrey, England	Some attempts at the immunotherapy of tumors in experimental animals
H. Altmann	5/29/68	Seibersdorf Reactor Center Vienna, Austria	Problems of radioresistance within the cell cycle
G. C. Alvino	5/22/68	Massachusetts Institute of Technology Cambridge, Massachusetts	Studies on nucleotide sequence of alanine tRNA from *E. coli*
Sonia R. Anderson	6/19/68	Biochemistry Division Department of Chemistry University of Illinois Urbana, Illinois	The reversible acid denaturation and hybridization of lactic dehydrogenase
Ettore Appella	5/29/68	National Cancer Institute National Institute of Health Bethesda, Maryland	Chemical structure of immunoglobulin light chains and its genetic implication
Wolfram Astertag	5/20/68	Department of Biophysics The Johns Hopkins University School of Medicine Baltimore, Maryland	Chemical mutagenesis in human cell cultures
John Bendler	9/19/68	Department of Microbiology University of Pennsylvania Philadelphia, Pennsylvania	Physical mapping functions in *Haemophilus influenzae* transformation
I. H. Billick	3/21/68	National Bureau of Standards Washington, D.C.	Analytical ultracentrifugation at variable angular velocity
Robert Bird	7/29/68	Physics Department Kansas State University Manhattan, Kansas	The rate of travel of the replication fork at different growth rates in *E. coli* 15T-
Richard Braun	10/15/68	Department of Radiology Division of Radiation Biology Case Western Reserve University Cleveland, Ohio	Time sequence of DNA application in the mitotic cycle
Charles Brinton	3/18/68	Department of Biophysics and Molecular Biology University of Pittsburgh Pittsburgh, Pennsylvania	Role of F-pili for nucleic acid transfer during conjugation and phage infection
Douglas Campbell	7/25/68	Department of Genetics University of Washington Seattle, Washington	X-ray enhanced genetic recombination in bacteriophage T4
D. L. D. Caspar	10/17/68	Children's Cancer Research Foundation Boston, Massachusetts	Symmetry and biomolecular form
Ruggero Ceppellini	10/16/68	Instituto di Genetica Dell Universita di Torino Torino, Italy	Genetics of immunoglobulins

SPEAKER	DATE	AFFILIATION	SUBJECT
Joe Cherry	10/18/68	Department of Horticulture Purdue University Lafayette, Indiana	Effects of X-rays on nucleic acid and protein synthesis in plants
A. P. Dalmasso	6/11/68	Instituto de Biología y Medicina Experimental Buenos Aires, Argentina	Complement-membrane interaction
Christiane Dosne de Pasqualini	9/17/68	Instituto de Investigaciones Hematologicas Buenos Aires, Argentina	Induction of murine leukemia by human lymphomas
Yukio Doida	2/22/68	The University of Rochester School of Medicine and Dentistry Rochester, New York	Comparative study of chromosome aberrations induced by high concentration of TdR and X-ray and its recovery
William Donachie	10/24/68	MRC Microbial Genetics Research Unit Department of Molecular Biology Edinburgh, Scotland	Control of DNA replication and cell division in *E. coli*
R. P. Donahue	6/6/68	Division of Medical Genetics The Johns Hopkins Hospital Baltimore, Maryland	Mouse oocyte maturation *in vitro*
F. A. Eiserling	10/7/68	Department of Bacteriology University of California Los Angeles, California	Fine structure of viruses
N. A. Evans	9/11/68	Department of Soils and Plant Nutrition University of California Berkeley, California	Inactivation of tobacco mosaic virus RNA by ultraviolet radiation
W. G. Flamm	8/8/68	University of North Carolina Durham, North Carolina	Highly repetitive sequences in mammalian DNA
Seymour Fogel	10/21/68	Department of Biology Brooklyn College Brooklyn, New York	Recent studies on meiotic gene conversion
P. G. Gahan	8/16/68	Department of Biology and Cell Science Woolwich Polytechnic London, England	Extranuclear DNA
H. Galjaard	8/30/68	Department of Cell Biology Rotterdam Medical Faculty The Netherlands	Microchemical and autoradiographic studies on intestinal epithelium after low radiation doses
Julian Gross	7/15/68	Microbiology Genetics Research University Medical Research Council Edinburgh, Scotland	Temperature-sensitive mutants of *B. subtilis* defective in DNA replication
P. B. Hamilton	1/11/68	Alfred I. du Pont Institute of the Nemours Foundation Wilmington, Delaware	Adventures in the ion-exchange chromatography of amino acids
Walter Harm	5/28/68	Division of Biology Southwest Center for Advanced Studies Dallas, Texas	New results on V-gene repair and photoenzymic repair in *Escherichia coli* cells
R. E. Harrington	11/25/68	University of California Davis, California	Properties of a high molecular weight DNA-protein complex from flow birefringence and viscosity measurements
Felix Haurowitz	1/18/68	Department of Chemistry Indiana University Bloomington, Indiana	Studies on antibody structure

SPEAKER	DATE	AFFILIATION	SUBJECT
Ulrich Heber	8/9/68	University of Düsseldorf Institute of Botany Germany	The membrane concept of frost injury and hardiness
R. D. Heidenreich	10/1/68	Bell Telephone Laboratories Incorporated Murray Hill, New Jersey	Electron diffraction and imagery
Egon Hidregi	5/23/68	Department of Pharmacology Baylor University College of Medicine Texas Medical Center Houston, Texas	The early effect of X-irradiation on liver ribosomal system *in vivo*
Sergei Georgievich Inge-Vechomor	5/6/68	Department of Genetics and Breeding Kiror Leningrad State University Leningrad, Russia	Complementation in the adenine-2 locus of Saccharomyces
F. P. Inman	10/29/68	Department of Microbiology University of Georgia Athens, Georgia	Biochemical studies of the subunits of γM-immunoglobulins
R. B. Inman	10/16/68	Biophysics Laboratory University of Wisconsin Madison, Wisconsin	The study of DNA base sequence, replication and deletion mutation by electron microscopy
Martin Kamen	5/24/68	Department of Chemistry University of Chicago Chicago, Illinois	Redox parameters in bacterial photosynthesis
G. W. Kidder	3/4/68	Department of Biology Amherst College Amherst, Massachusetts	Biosynthesis of pteridines
Sung-hou Kim	9/12/68	Department of Biology Massachusetts Institute of Technology Cambridge, Massachusetts	Structural aspects of base pairing
A. Kleinschmidt	10/14/68	Department of Biochemistry New York University School of Medicine New York, New York	Conformational studies of viral nucleic acids by electron microscopy
P. C. Koller	9/13/68	Chester Beatty Research Institute London, England	The cellular basis of immunological competence
Sohei Kondo	2/9/68	Department of Fundamental Radiology Faculty of Medicine Osaka University Kita-ku, Japan	Mutagenicity versus radiosensitivity in *E. coli*
M. S. Legator	7/30/68	Chief, Cell Biology Branch Division of Nutrition Food and Drug Administration Washington, D.C.	Coordinated biological screens for characterizing chemical mutagens
M. D. Lilly	9/18/68	Department of Chemical Engineering University College London London, England	Large-scale enzyme isolation
P. C. Loh	2/14/68	National Institute of Allergy and Infectious Diseases National Institutes of Health Bethesda, Maryland	Some aspects of the cytopathogenicity of Reovirus Type 2
C. B. Lozzio	2/26/68	Memorial Research Center The University of Tennessee Knoxville, Tennessee	Radiosensitivity of ELD ascites tumor cells at different stages of the cell cycle
Harvey Lyman	10/25/68	Biology Department State University of New York Stony Brook, Long Island, New York	Role of light in chloroplast DNA replication and repair

SPEAKER	DATE	AFFILIATION	SUBJECT
Inga Mahler	7/18/68	Graduate Department of Chemistry Brandeis University Waltham, Massachusetts	Transformation of UV-sensitive mutants of *Micrococcus lysodeikticus*
John H. Manley	7/23/68	Los Alamos Scientific Laboratory Los Alamos, New Mexico	Electron chemistry and collagen structure
Guglielmo Marin	11/4/68	International Laboratory of Genetics and Biophysics Naples, Italy	Reversion of polyoma virus-induced transformation in hamster cells
H. Matthei	12/11/68	Max-Planck Institut für Experimentelle Medizin Göttingen, Germany	New phenomena observed in cell-free study of gene expression in bacterial and human systems
H. D. Mennigmann	9/9/68	Institut für Mikrobiologie Universität Frankfurt Frankfurt, Germany	The lesions responsible for the sensitized inactivation of *E. coli* by black light
Kin-ichiro Miura	8/21/68	Nagoya University Nagoya, Japan	Functional sites of tRNA
Marylin Monk	7/15/68	Microbiology Genetics Research University Medical Research Council Edinburgh, Scotland	Studies on indirect induction of prophage λ
Pablo Mori-Chavez	11/13/68	Laboratorio de Investigacion de Cancer Lima, Peru	The influence of high altitude on neoplastic growth
D. J. Morre	10/4/68	Department of Botany and Plant Pathology Purdue University Lafayette, Indiana	Golgi-apparatus: structure and function
K. H. Muench	6/17/68	School of Medicine University of Miami Miami, Florida	Interaction of chloroquine and tRNA
K. H. Muench	6/18/68	School of Medicine University of Miami Miami, Florida	Evidence for subunits in Prolyl-tRNA synthetase of *E. coli*
A. H. Nishikawa	6/21/68	Department of Biochemistry and Biophysics Oregon State University Corvallis, Oregon	Polyvalyl RNase aggregation: model for hydrophobic interactions
Nic Odartchenko	8/29/68	Swiss Institute for Experimental Cancer Research Lausanne, Switzerland	Cytokinetic studies on two types of "end cells" in mammalian somatic tissues: erythropoiesis and lymphopoiesis
E. J. Ofengand	2/21/68	Department of Biochemistry University of California San Francisco, California	Studies on the function of pseudouridine in tRNA
George Palade	10/11/68	The Rockefeller University New York, New York	Function of the endoplasmic reticulum
C. R. Parks	4/1/68	Department of Botany University of North Carolina Chapel Hill, North Carolina	A method of flavinoid characterization for taxonomic purposes and its application in a study of the genus Gossypium
Ishwari Prasad	5/3/68	Department of Biochemistry and Microbiology Rutgers – The State University New Brunswick, New Jersey	Biological assay of the mutagenic activity of pesticides and their residues

SPEAKER	DATE	AFFILIATION	SUBJECT
Efraim Racker	10/9/68	Department of Biochemistry and Molecular Biology Cornell University Ithaca, New York	Function and structure of the inner mitochondrial membrane
Harry Rappaport	12/5/68	Department of Biology Temple University Philadelphia, Pennsylvania	New methods for the purification of tRNA's and tRNA synthetases
Michael Reedy	10/3/68	University of California Los Angeles, California	X-ray and electron microscope studies on the mechanism of cross-bridge action in striated muscle
M. A. Resnick	9/16/68	Donner Laboratories University of California Berkeley, California	Genetic control of radiation-induced lethality and mutation in yeast
Emanuel Riklis	8/15/68	Nuclear Research Center – Negev Atomic Energy Commission Beersheba, Israel	Photoproducts of thymine
G. Ruhenstroth	9/10/68	Max-Planck Institut für Biochemie Munich, Germany	Two populations of small lymphocytes
Joseph Sambrook	11/20/68	The Salk Institute San Diego, California	The state of viral DNA and transformed cells
Ioannis Scarpa	2/12/68	Department of Chemistry Northwestern University Evanston, Illinois	Slow hydrogen-deuterium exchange in a non-α-helical polymide
H. E. Schaffer	5/13/68	Department of Genetics North Carolina State University Raleigh, North Carolina	Chromosome segregation
Jack Schubert	8/2/68	Radiation Health Division Graduate School of Public Health University of Pittsburgh Pittsburgh, Pennsylvania	Irradiated sucrose and peroxides
Verne Schumaker	4/23/68	Department of Chemistry University of California Los Angeles, California	Review of basic studies on sedimentation at UCLA
J. H. Schwartz	8/20/68	Department of Microbiology New York University Medical Center New York, New York	A role for bacteriophage complementary strand RNA in cell-free protein synthesis
S. Seno	9/11/68	Okayama University Medical School Okayama, Japan	Erythroid cell specialization: Heme and RNA synthesis in early denucleated cells
R. P. Singhal	7/1/68	All-India Institute of Medical Sciences New Delhi, India	RNA fingerprints of cellular fractions from cancer cells
Fred Snyder	2/1/68	Medical Division Oak Ridge Associated Universities	Glycerol ethers in normal and neoplastic tissue
J. M. Sowinski	1/30/68	The Pharmacia Company St. Louis, Missouri	Techniques in gel-filtration
A. H. Sparrow	5/10/68	Brookhaven National Laboratory Upton, New York	The relationship between chromosome variables and radiosensitivity
Gabriel Stein	8/2/68	Department of Physical Chemistry The Hebrew University Jerusalem, Israel	Inactivation of some enzymes and bacteriophage by radiation and by H atoms

SPEAKER	DATE	AFFILIATION	SUBJECT
F. C. Steward	12/11/68	Division of the Laboratory for Cell Growth and Development Division of Biological Sciences Cornell University Ithaca, New York	Growth induction in cultured cells: Some controversies, some current conclusions
B. L. Strehler	11/12/68	Department of Biological Sciences University of Southern California Los Angeles, California	The molecular genetics of aging
Betsy M. Sutherland	11/22/68	Walter Reed Army Institute of Research Washington, D.C.	Mechanisms of inhibition of pyrimidine dimer formation in DNA by acridine dyes
D. T. Suzuki	5/27/68	Department of Zoology The University of British Columbia Vancouver, Canada	Temperature-sensitive lethal mutations in Drosophila
J. J. Trentin	11/16/67	Baylor Medical School Baylor University Waco, Texas	Results of caesarean derivation and barrier maintenance of a large inbred mouse colony
Pieter van de Putt	9/3/68	Rijswijk, The Netherlands	Genetic control of radiation sensitivity
Jack Vant Hof	5/24/68	Brookhaven National Laboratory Upton, Long Island, New York	Regulation of cell division and DNA synthesis in excised plant tissue
†E. P. Wigner	3/26/68	Palmer Physical Laboratory Princeton University Princeton, New Jersey	Are we machines?
J. W. T. Wimpenny	8/12/68	Department of Microbiology University of College of South Wales in Monmouthshire Cardiff, Wales	Oxygen regulation in facultative metabolism
V. P. Whittaker	4/4/68	Department of Biochemistry University of Cambridge Cambridge, England	The biochemistry of snyaptosomes
Elizabeth Work	9/9/68	Twyford Laboratories London, England	Cell walls of *Micrococcus radiodurans*
H. Yamaguchi	7/26/68	Tokyo University Tokyo, Japan	Metabolic restitution of chromosome breaks after irradiation
Michael Yarmolinsky	11/7/68	National Institutes of Health Bethesda, Maryland	The integration and excision of the bacteriophage lambda genome
A. F. Yanders	3/22/68	School of Natural Sciences Michigan State University East Lansing, Michigan	Visible polarity and meiotic drive in Drosophila

†Joint Biology, Chemistry, and Physics Division Seminar.

UNIVERSITY OF TENNESSEE–OAK RIDGE GRADUATE SCHOOL OF BIOMEDICAL SCIENCES–BIOLOGY DIVISION SPECIAL LECTURE SERIES

During 1968, the following visiting scientists presented lectures under the special lecture series begun in 1967 under the joint sponsorship of the University of Tennessee–Oak Ridge Graduate School of Biomedical Sciences and the Biology Division:

P. D. Boyer	10/18/68	Molecular Biology Institute University of California Los Angeles, California	Some isotopic probes of enzyme mechanisms
J. B. Gall	9/26/68	Department of Biology Yale University New Haven, Connecticut	Differential gene replication
Jerard Hurwitz	2/8/68	Albert Einstein College of Medicine Yeshiva University Bronx, New York	Reactions leading to repair of DNA
N. E. Miller	3/8/68	Rockefeller University New York, New York	Glandular and visceral learning
Fred Richards	12/12/68	Molecular Biophysics Department Yale University New Haven, Connecticut	The tertiary structure of proteins
Herbert Stern	7/16/68	Department of Biology University of California La Jolla, California	Biochemical studies on meiosis
J. E. Till	1/11/68	Division of Biological Research The Ontario Cancer Institute Toronto, Canada	Cellular differentiation in the hemopoietic system
Gregorio Weber	5/16/68	Department of Chemistry and Chemical Engineering University of Illinois Urbana, Illinois	Use of energy transfer methods in protein ligand equilibrium

SCHEDULED SPEAKERS AT PROFESSIONAL MEETINGS IN 1969

Papers by members of the Biology Division will be presented at the following professional society meetings during 1969. Names of speakers appear in boldface. (Listing includes information received up to February 28, 1969.)

January 25–28

8th Annual Midwinter Conference of Immunologists, Pasadena, California – **Takashi Makinodan**

February 3–5

American Society of Plant Physiologists (Southern Section), Mobile, Alabama – **A. H. Haber,** D. E. Foard, and Stella W. Perdue

February 11–13

Ciba Foundation Symposium on Mutation as Cellular Process, London, England – **R. F. Kimball**; and **W. L. Russell**

February 26–28

13th Annual Meeting of the Biophysical Society, Los Angeles, California – **P. A. Swenson** and R. B. Setlow; **J. E. Donnellan, Jr.,** and R. S. Stafford; **J. W. Longworth**; **R. O. Rahn** and L. C. Landry; **M. L. Randolph** and Jane K. Setlow; **Peter Mazur,** S. P. Leibo, John Farrant, E. H. Y. Chu, and M. G. Hanna, Jr.; **S. P. Leibo** and Peter Mazur; **R. B. Setlow,** W. L. Carrier, and Jane K. Setlow; and **J. A. Knopp,** J. W. Longworth, and R. O. Rahn

March 5–7

23rd Annual Symposium on Fundamental Cancer Research, Houston, Texas – **G. David Novelli**; and **Jane K. Setlow**

March 11–15

International Academy of Pathology, San Francisco, California – **R. C. Brown**, C. B. Richter, and M. D. Bloomer

March 23–25

60th Annual Meeting of the American Association for Cancer Research, San Francisco, California – **R. C. Brown**, Vincenzo Covelli, A. C. Upton, and Lou C. Satterfield; **R. L. Tyndall** and J. A. Otten; and **N. K. Clapp** and A. W. Craig

March 28–29

American Society of Microbiologists, Clemson, South Carolina – **John M. Boyle**

March 31–April 3

Symposium on Protein–Nucleic Acid Interaction (22nd Annual Biology Research Conference), Gatlinburg, Tennessee – **G. David Novelli**; and **Audrey Stevens**

April 7–11

6th Meeting of the Federation of European Biological Societies, Madrid, Spain – **H. G. Gassen** and Mayo Uziel

April 8–11

4th International Symposium of the International Committee on Laboratory Animals, Washington, D.C.– **C. B. Richter**, R. W. Tennant, and M. L. Simmons; L. **J. Serrano**, R. C. Allen, R. C. Brown, N. K. Clapp, G. E. Cosgrove, E. B. Darden, Jr., T. T. Odell, Jr., R. L. Tyndall, A. C. Upton, and H. E. Walburg, Jr.; and **M. L. Simmons**, C. B. Richter, and R. W. Tennant

April 10–12

Annual Meeting of the Tennessee Medical Association and the Tennessee Society of Pathologists, Gatlinburg, Tennessee – **R. C. Brown**

April 13–18

Federation of American Societies for Experimental Biology, Atlantic City, New Jersey – **J. F. Albright** and T. F. Omer; **M. L. Davis**; **S. A. Fairfield**, C. J. Pennington, Jr., and W. E. Barnett; **Wallace Friedberg**, B. R. Neas, D. N. Faulkner, and C. C Congdon; **Joan Wright Goodman**, Sarah G. Shinpock, and Chareen G. Grubbs; **F. C. Hartman**; **V. K. Jenkins**, H. E. Walburg, Jr., A. C. Upton, and Lou C. Satterfield; **Kai-Lin Lee**; **H. O. Weeren**, C. W. Hancher, A. D. Ryon, E. F. Phares, A. D. Kelmers, and G. David Novelli; **E. H. Perkins**, Takashi Makinodan, and A. C. Upton; **M. R. Proffitt** and C. C Congdon; **Joan L. Rasor**; **P. O. Ritter**, F. J. Kull, and K. Bruce Jacobson; **L. R. Shugart** and M. P. Stulberg; **Mayo Uziel** and H. G. Gassen; **W. D. Wicks**; **J. L. Wittliff** and F. T. Kenney; and **W. K. Yang**, K. M. Hilse, and R. A. Popp

April 28–May 2

International Atomic Energy Agency Symposium on Radiation Carcinogenesis, Athens, Greece – A. C. Upton, R. C. Brown, J. W. Conklin, G. E. Cosgrove, E. B. Darden, Jr., M. A. Kastenbaum, T. T. Odell, Jr., L. J. Serrano, R. L. Tyndall, and **H. E. Walburg, Jr.**

April 27–May 1

54th Annual Meeting of the American Association of Cereal Chemists, Chicago, Illinois – **J. X. Khym**, R. L. Jolley, and C. D. Scott

May 4–9

69th Meeting of the American Society for Microbiology, Miami Beach, Florida – **J. A. Fralick**, W. D. Fisher, and H. I. Adler; **R. K. Fujimura**; **R. T. Jones** and Roy Curtiss III; **N. A. Nugent** and R. C. Fuller; and **R. W. Tennant**

May 6–8

Symposium on Radiation Repair (2nd International Conference on Radiosensitivity and Radiosensitizing Drugs), Rome, Italy – **J. D. Regan**

May 18–22

17th Annual Meeting of the Radiation Research Society, Cincinnati, Ohio – **M. A Bender**; **E. B. Darden, Jr.**, N. K. Clapp, A. C. Upton, R. S. Bender, and M. C. Jernigan; **M. L. Davis** and C. C Congdon; **T. T. Odell, Jr.**, E. A. Burch, Jr., C. W. Jackson, and T. J. Friday; **L. H. Smith** and T. W. McKinley, Jr.; **A. C. Upton**, R. C. Brown, J. W. Conklin, G. E. Cosgrove, E. B. Darden, Jr., M. A. Kastenbaum, T. T. Odell, Jr., L. J. Serrano, R. L. Tyndall, and H. E. Walburg, Jr.; and **R. C. von Borstel** and R. H. Smith

June 8–11

AtoMedic '69 – An Atomic Energy Commission Briefing for Science Writers, Brookhaven National Laboratory, Upton, New York – **C. C Congdon**; and **J. D. Regan**

August 11–15

2nd International Conference on Medical Physics, Boston, Massachusetts – **J. F. Spalding**, Diana M. Popp, and R. A. Popp

Foreign Travel

As part of its continuing effort to promote international scientific communication, the Biology Division sponsored visits to 18 foreign countries in 1968. The activities of Division members traveling abroad last year ranged from participation in conferences and meetings to presenting invited seminars at research institutes, colleges, and universities.* Countries visited were:

Europe –	Denmark	**Asia** –	Japan
	England		Taiwan
	France		
	Germany		
	Ireland		
	Italy		
	The Netherlands	**The Americas** –	Brazil
	Norway		Canada
	Russia		Chile
	Switzerland		Mexico
	Wales		Venezuela

Europe

Waldo E. Cohn went to Bellagio, Italy, June 10 to attend a meeting of the Combined Commission on Biochemical Nomenclature (CBN) of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry. Dr. Cohn is currently serving as Secretary of CBN.

In June and July, **C. C Congdon** traveled in Italy, England, and Russia. He presented a seminar at Casaccia, Rome, May 22–24, and then went to Padova, Italy, for the IInd International Conference on Germinal Centers of Lymphatic Tissue, held June 25–28, where he presented a paper. He was joined there by **M. G. Hanna, Jr.**, and **Paul Nettesheim**, who also presented papers. After a visit to The Medical School in Birmingham, England, July 1–3 to discuss collaborative research on radiation chimeras with Dr. Brynmor Thomas, Dr. Congdon traveled to Moscow for the IAEA Panel Meeting on Bone Marrow Cell Transplantation, held June 21–26. Drs. Hanna and Nettesheim, following the Conference on Germinal Centers, visited a number of European laboratories specializing in carcinogenesis, such as the Institute of Histologic Anatomy, University of Perugia Medical School, Perugia, Italy, and the Institute of Tobacco Research, Hamburg, Germany.

*Titles of papers and lectures given abroad appear in the list of **LECTURES**, pp. 14–36.

Dorothy M. Skinner flew to Paris in September to present a paper at the International Congress of Embryology, held September 2–7. While in Paris, Dr. Skinner collaborated with Dr. C. Tchernigovtzeff on research on the crustacean molting cycle.

R. C. Fuller was in Freudenstadt, Germany, June 2–8, to attend the First International Congress of Photosynthesis and to present a paper at the Plenary Session. He then went to Cardiff, Wales, to confer with Drs. David Hughes, Julian Wimpenny, and Geoffrey Callely at the University of Wales. Dr. Fuller also attended and presented a paper at the International Conference on Photosynthesis, held June 12–14 in London, England.

Alexander Hollaender went to Berlin, Germany, in late February to give a paper at a meeting of the German Biophysical Society. During July, Dr. Hollaender traveled in Italy, Germany, and The Netherlands. His main purpose was to visit the various Italian laboratories in his continuing role as consultant to the Comitato Nazionale per l'Energia Nucleare. He spent July 4–5 in Milan, where he discussed problems in radiation biology with colleagues at the University of Milan and problems in genetics with L. L. Cavalli-Sforza at the University of Pavia. Dr. Hollaender was in Rome and Naples the following week. In Rome, he conferred with personnel of the Animal Radiobiology Laboratory, Casaccia, on biological research there, and with Carlo Polvania and others at the Comitato Nazionale per L'Energia Nucleare on the overall program for radiation biology in Italy; in Naples, he talked with A. A. Buzzati-Traverso about the expansion of Italy's research facilities. Traveling then to Germany, Dr. Hollaender discussed mutation production by chemicals with Friedrich Vogel and G. Röhrborn at the University of Heidelberg. Before returning to Oak Ridge, he spent a few days in Amsterdam, The Netherlands, where he discussed plans for the 1970 International Congress of Radiation Research with F. H. Sobels.

In October, Dr. Hollaender made a trip abroad which included visits to Paris, Vienna, and London. After visiting French scientists in Paris, October 16–20, to obtain records for the University of Tennessee Archives for Radiation Biology, he went to Vienna to present a paper at a panel meeting of the International Atomic Energy Agency. Dr. Hollaender then stopped in London to visit British scientists and collect literature on radiation biology.

R. F. Kimball traveled in England, France, and Holland during May and June. He went first to Paterson Laboratories in Manchester, England, May 28–29, to confer with Drs. Alma Howard and Michael Ebert about problems in radiation biology and to present a seminar. On May 29, he went to London to discuss mutual problems with Dr. Tikvah Alper at the Experimental Radiopathology Research Unit, MRC. Later he spoke on repair of mutation induction at the University of London and participated in a small conference of the Photobiology Group and the Radiobiology Section at the British Institute of Radiology. Dr. Kimball then went to Leiden, Holland, to consult with Professor F. H. Sobels, president of the IARR, on Association business and on radiation mutagenesis. On June 3, he traveled to Interlaken, Switzerland, to attend meetings of the IARR Council, of which he is Secretary-Treasurer, and the European Society for Radiation Biology.

F. T. Kenney was a traveler to the British Isles in September. At Mill Hill, London, he conferred with Dr. J. R. Tata of the National Institute for Medical Research on hormonal regulation of RNA and protein synthesis. At the University of Nottingham, Nottingham, he gave a paper in a symposium on Hormones in Development, sponsored by the National Foundation and held September 9–12. Dr. Kenney then went to Dublin, Ireland, to discuss control mechanisms with Drs. B. Spencer and F. Winder at Trinity College.

H. V. Malling visited several research installations in Denmark and Norway from February 17 to March 3, discussing problems of mutual interest with investigators at the University of Århus, Århus, Denmark; the University of Oslo, Oslo, Norway; and the Food and Drug Department, Kobenhavns Sundhedsstyrelse, Copenhagen, Denmark.

John Papaconstantinou and **Tuneo Yamada** presented papers at the International Symposium on The Biochemistry of the Eye, held June 24–28 at the University of Nijmegen, The Netherlands. Before the Symposium, Dr. Yamada visited the University of Camerino, Camerino, Italy, and the Naples Zoological Station, where he discussed biochemical studies of lens regeneration with staff members. He gave a seminar at both Camerino and Naples. In late summer, he presented a paper and chaired a session at the Sixth International Congress of Embryology, held in Paris, August 31–September 7. He then visited the Biology Department of the University of Cologne, where he discussed the problem of embryonic induction with Drs. W. Vahs, A. G. Johnen, and H. Englander, and presented a seminar.

R. O. Rahn attended a NATO International Summer Institute on the Quantum Theory of Chemical Reactivity of Excited Organic Molecules – Biochemical Application. The Institute was held in Menton, France, July 1–24.

A. C. Upton was in London, England, January 7–13 to participate in a meeting of the International Commission on Radiological Protection.

R. A. Weisberg traveled widely in Europe during March and April. In Naples, Italy, he attended a workshop on lysogeny (March 28–31) sponsored by the Italian National Research Council and the European Molecular Biology Organization; afterwards he conferred with colleagues at the International Laboratory of Genetics and Biophysics about the possibility of collaborative research and possible future experiments. Dr. Weisberg then flew to Geneva, Switzerland, for a visit to the Institute of Molecular Biology and discussion with members of the staff. Before returning to the Division, he paid similar visits to the Pasteur Institute, Paris, France, and the MRC-Microbial Genetics Research Unit, London, England.

Asia

F. J. de Serres and **R. C. von Borstel** were in Tokyo, Japan, May 13–21, to present papers at the 11th Plenary Meeting of the Committee on Space Research.

M. A Bender, E. H. Y. Chu, F. J. de Serres, H. V. Malling, and **R. C. von Borstel** each gave a paper at the XIIth International Congress of Genetics, held August 19–28 in Tokyo, and **R. F. Kimball** chaired a small symposium. Drs. Bender, de Serres, and von Borstel also presented papers at the International Symposium on Genetic Effects of Space Environment, held in Tokyo, August 25, in conjunction with the Genetics Congress, and Dr. Kimball served as chairman of one of the sessions.

While in Japan, Dr. Bender traveled to Sapporo to present a paper at the Symposium on Chromosomes and Related Problems. Afterwards he visited the cities of Kyoto and Nara, and Mie Prefecture, for discussions with colleagues.

Before the Genetics Congress, Dr. Chu was in Taipei, Taiwan, to present lectures, July 17–August 13, in a Summer Science Seminar at the Academia Sinica and to deliver a paper at a genetics symposium held August 13–15.

After the Tokyo Congress, Drs. Kimball, de Serres, Malling, and von Borstel traveled to Kyoto to participate in the International Symposium on Genetic Effects of Radiation and Radiomimetic Chemicals. Dr. Kimball remained in Japan for a few days after the symposium to discuss radiation genetics with investigators at various universities and research institutes. Before returning to the Division, Dr. von Borstel attended a conference of yeast geneticists held in Osaka September 2–5.

F. P. Conte flew to Tokyo in December to participate in a National Science Foundation symposium on Endocrine Glands and Osmoregulation in Fishes. Dr. Conte was cochairman of the session on Cellular and Subcellular Aspects of Osmoregulatory Adjustments.

The Americas

J. L. Epler, W. D. Fisher, W. D. Gude, and **Mayo Uziel** participated from August 30 to September 7 as lecturers in a special training course ("Macromolecular Separations") held in Caracas, Venezuela, at the Venezuelan Institute for Scientific Investigation. Dr. Epler also gave a research seminar at Central University in Caracas.

Alexander Hollaender spent several weeks in South America during November and December. Before attending the Symposium on Nuclear Physiology and Differentiation in Belo Horizonte, Brazil, he met with staff members of the University of Chile, Santiago, Chile, to discuss plans for a symposium on Processing in the Vertebrate Visual System to be held at the University in 1970. He then went to São Paulo, Brazil, where he met with members of the Oceanographic Institution of the University of São Paulo to work out details for the Symposium on Fertility of the Sea scheduled for São Paulo in 1969. After the Belo Horizonte meeting, Dr. Hollaender traveled to the University of Bahia, Salvador, Brazil, to survey the current state of biological research there and to formulate plans for future scientific meetings. He returned to Oak Ridge on December 17.

J. W. Longworth, R. O. Rahn, and **R. B. Setlow** attended and gave papers at the International Symposium on Basic Mechanisms in Photochemistry and Photobiology, held in Caracas, Venezuela, December 4–8, 1967.

Takashi Makinodan was in Caracas, Venezuela, January 18–20, to present two invited lectures at the Venezuelan Institute for Scientific Investigation and to confer with Dr. Sergio Arias-Cazorla.

Ellen Mattingly traveled to São Paulo, Brazil, in the fall. From September 12 to October 11, Dr. Mattingly was at the University of São Paulo to consult and conduct research with Dr. Francisco Lara and his colleagues on problems in dealing with *Rhynchosciara angelae.* While in Brazil, she also visited the University of Belo Horizonte, the University of Brasilia, and the Medical School in Ribero Prêto.

O. L. Miller, Jr., Elliot Volkin, and **R. C. von Borstel** gave papers at the Symposium on Nuclear Physiology and Differentiation, held December 1–6 in Belo Horizonte, Brazil, at the University of Minas Gerais. **Alexander Hollaender,** as President of the symposium, delivered the introductory and concluding remarks. Dr. Miller gave a seminar at the Institute of Scientific Investigations in Caracas, Venezuela, before attending the symposium; and, while in Brazil, visited laboratories in Rio de Janeiro and Porto Alegre. He traveled to Argentina (Buenos Aires and La Plata) for discussions with colleagues before returning to Oak Ridge. Dr. von Borstel, after the Belo Horizonte meeting, paid a short visit to the Institute of Biophysics in Rio de Janeiro.

W. D. Wicks, during December, lectured and directed laboratory sessions in an advanced course in biochemistry held at the University of Pernambuco, Recife, Brazil. He visited laboratories in São Paulo and Rio de Janeiro before returning to Oak Ridge.

E. F. Oakberg, J. R. Reel, W. D. Wicks, and **J. L.Wittliff** attended the International Congress of Endocrinology in Mexico City, June 30–July 5. Drs. Oakberg and Reel presented papers. While in Mexico, Dr. Wicks presented seminars at the University of Mexico, Mexico City; at the University of San Luis Potosi, San Luis Potosi; and at the University of Nuevo Leon, Monterrey.

R. C. Allen was in Toronto, Canada, December 2–5, 1967, to participate in the 10th annual meeting of The American Society of Hematology.

J. N. Dumont presented a seminar to the Zoology Department of the University of Windsor, Windsor, Ontario, Canada, November 28–30, 1967.

B. E. B. Moseley paid a brief visit to the Chalk River Laboratories of Atomic Energy of Canada, Ltd., in Chalk River, Ontario, on May 3.

G. D. Novelli was in Hamilton, Ontario, Canada, October 23–24 to present a seminar at McMaster University.

R. A. Wallace spent December 2–4 at the Cancer Research Institute, Notre Dame Hospital, Montreal, Canada, doing collaborative research with Dr. Shuichi Karasaki.

Meetings and Conferences

1968

PROCEEDINGS OF THE TWENTY-FIRST ANNUAL BIOLOGY RESEARCH CONFERENCE

The proceedings of the twenty-first annual Biology Research Conference (**Molecular Aspects of Differentiation**), held at Gatlinburg, Tennessee, April 8–11, 1968, were published as a supplement to the October issue of the *Journal of Cellular Physiology.* Listed below is the table of contents:

John Papaconstantinou and Emilia M. Julku	The regulation of ribosomal RNA synthesis and ribosomal assembly in the vertebrate lens
Klaus Scherrer and Lise Marcaud	Messenger RNA in avian erythroblasts at the transcriptional and translational levels and the problem of regulation in animal cells
PANEL DISCUSSION	Present status and perspectives in the study of cytodifferentiation at the molecular level
C. L. Markert, leader	Initial remarks
P. R. Gross, W. J. Rutter, D. L. Kimmel, Jr., and D. L. Nanney, panel members	Discussion

AEC-NCI COCARCINOGENESIS REVIEW

A group of 23 scientists representing the National Cancer Institute and the U.S. Atomic Energy Commission visited the Biology Division June 17–18 to review the AEC-NCI Cocarcinogenesis Program.

Visitors were Carl G. Baker, Howard E. Bond, Charles W. Boone, Michael A. Chirigos, James Culp, James T. Duff, Hans L. Falk, Melvin S. Fish, Maurice S. Fortin, Alfred Hellman, Timothy E. O'Connor, Herbert J. Rapp, Frank J. Rauscher, Umberto Saffiotti, Jesse Steinfeld, Joseph Valego, and John M. Venditti, all of the NCI; C. W. Edington, AEC; Robert J. Huebner, National Institute of Allergy and Infectious Diseases; Bruce H. Smith, Armed Forces Institute of Pathology; Murray M. Copeland, M. D. Anderson Hospital and Tumor Institute; Joseph L. Melnick, Baylor University College of Medicine; and Jane C. Wright, New York Medical College.

An abstract booklet covering some of the Cocarcinogenesis projects was prepared for the visitors, and the following investigators discussed their work:

G. David Novelli	Changes in some transfer RNA's in cultured L-M cells when grown in the mouse
F. T. Kenney	Regulation of enzyme synthesis in liver and hepatoma
Stanfield Rogers	Viruses and the potential therapy of cancer and genetic deficiency diseases
H. V. Malling	The relationship between carcinogenicity and mutagenicity of methylating compounds
E. H. Y. Chu	Chemical mutagenesis in cultured mammalian cells
D. G. Doherty	Inhalation carcinogenesis program. (*a*) Experimental plan and inhalation procedures
M. G. Hanna, Jr.	Inhalation carcinogenesis program. (*b*) Analysis of current data
Paul Nettesheim	Inhalation carcinogenesis program. (*c*) Persistence of antigens during the immune response
A. C. Upton	Mammalian studies on carcinogenesis by chemicals, viruses, and radiation
R. B. Setlow	Energy transfer, molecular conformation, and repair
N. G. Anderson	Summary of the MAN Program

BIOMEDICAL PROGRAM DIRECTORS MEETINGS

During 1968, representatives of the Biology Division attended three meetings of the Biomedical Program Directors of the U.S. Atomic Energy Commission. These meetings are held at Commission laboratories each year and are designed to review research in the various biomedical programs supported by the AEC Division of Biology and Medicine.

R. F. Kimball, Director of the Biology Division, attended the winter meeting at the Argonne Cancer Research Hospital in Chicago, Illinois, February 12–13; and both he and J. L. Liverman, an Assistant Director of the Oak Ridge National Laboratory, attended the spring meeting in Rochester, New York, May 6–7. Dr. Kimball also went to the fall meeting at the Savannah River Laboratory in Augusta, Georgia, October 7–8.

CARCINOGENESIS–MOLECULAR BIOLOGY ROUND TABLE

A round-table workshop on carcinogenesis and how molecular biology may relate to its understanding was held June 24–28 at Woods Hole, Massachusetts. The meeting was organized by the Biology Division with the support and cooperation of the National Cancer Institute.

The workshop consisted of informal round-table discussions and a summary session. Thirty-one scientists took part in the discussions, and nine gave reviews at the summary session.

Participants from the Biology Division were: Alexander Hollaender, F. T. Kenney, J. L. Liverman, G. David Novelli, and A. C. Upton. Dr. Hollaender and Dr. Upton were among the speakers at the summary session.

ROUND TABLE ON MUTAGENESIS

In September the Biology Division sponsored a **Round Table on Mutagenesis** at Gaithersburg, Maryland; this meeting was prompted by a more informal discussion on the same subject which was held at the Division in April and attended by three outside investigators. About 40 biologists from all over the United States took part in the Gaithersburg meeting.

Discussion sessions were held on several aspects of chemical mutagenesis: basic chemical mechanisms, basic biological mechanisms, practical tests, metabolic and chemical studies, and monitoring of human populations. Questions on the criteria for mutagenesis in various organisms, on the chemical problems of mutagenic action, and on the necessary emphases for future research were discussed.

Members of the Division who attended were: E. H. Y. Chu, R. B. Cumming, F. J. de Serres, W. M. Generoso, Alexander Hollaender, R. F. Kimball, H. V. Malling, and W. L. Russell. Also attending was J. L. Liverman, an Assistant Director of the Oak Ridge National Laboratory.

FALL IMMUNOLOGY WORKSHOP

An annual **Fall Immunology Workshop**, intended primarily for immunologists in the Southeastern United States, was initiated this year. Organized by Takashi Makinodan, Biology Division; F. P. Inman, University of Georgia; and J. J. Vazquez, Duke Medical Center, this workshop was designed to provide information exchange in an informal atmosphere and to offer the opportunity for participants, especially pre- and postdoctoral fellows, to become familiar with the various institutes and immunologists in the Southeast.

The first meeting was held at the Oak Ridge Playhouse on October 17 and 18, and was attended by over 100 immunologists and students. An abbreviated program follows:

Welcoming Address – J. L. Liverman

Opening Remarks – R. F. Kimball

Immune Pathogenesis of Glomerulonephritis – Formal address by Frank J. Dixon, Department of Pathology, Scripps Clinic and Research Foundation, La Jolla, California

Antibody Structure – Review by Frank P. Inman, Department of Microbiology, University of Georgia; discussion moderated by Parker A. Small, Jr., Department of Microbiology, College of Medicine, University of Florida

Cellular Aspects of Immune Response – Review by Joseph F. Albright, Biology Division, Oak Ridge National Laboratory; discussion moderated by Jacinto J. Vazquez, Department of Pathology, Duke Medical Center

In future meetings, topics of discussion may include: immunogenetics, hypersensitivity and autoimmunity, phagocytosis, the mechanism of antibody synthesis and secretion, and somatic antigens.

SEVENTH CONFERENCE ON MAMMALIAN CYTOLOGY AND SOMATIC CELL GENETICS

In 1962 the Biology Division initiated conferences on **Mammalian Cytology and Somatic Cell Genetics**, which have since been held under various sponsorships. This year the Division was host to the seventh conference in the series, which was held at Gatlinburg, Tennessee, October 23–26. E. H. Y. Chu organized the meeting, and more than 150 investigators from the United States and Canada attended.

In the past these conferences have dealt with various aspects of human and mammalian genetics, especially the nature of normal and abnormal chromosomes. Sessions were held this year on human cytogenetics, chromosome cytology in various species, chromosome structure and somatic cell genetics, and induced chromosome abnormalities. About fifty papers were presented.

ANNUAL INFORMATION MEETING

The Oak Ridge National Laboratory Advisory Committee for Biology visited the Division November 18–20 for the **Annual Information Meeting**. The committee is composed of Rollin D. Hotchkiss, Rockefeller University; Henry S. Kaplan, Stanford University Medical School; C. L. Markert, Yale University; R. D. Owen, California Institute of Technology; Herschel Roman, University of Washington; and Earl R. Stadtman, National Heart Institute.

At the meeting Division investigators summarized their work for the advisory group, and Committee members visited individual laboratories and participated in informal discussions. An abbreviated program of speakers and topics follows:

Opening Remarks	
R. F. Kimball	
Biochemistry	
Mayo Uziel	The determination of nucleic acid sequences
W. E. Barnett	Translational apparatus of cellular organelles
F. T. Kenney	Hormonal regulation of liver enzyme synthesis
Biophysics	
J. R. Einstein	Structure of the thymine photodimer from UV-irradiated DNA
J. E. Donnellan, Jr.	Photochemistry and photobiology of bacterial spores
Immunology	
Takashi Makinodan	Cellular aspects of immune response and aging
Carcinogenesis	
Stanfield Rogers	The regulation of the blood level of specific metabolites through use of chronic dialysis against specific enzymes
M. G. Hanna, Jr.	The significance of antigen localization in the immune reaction
Genetics	
Jane K. Setlow	Repair of DNA in *Hemophilus influenzae*
E. H. Y. Chu	Mutation induction in cultured mammalian cells
J. G. Brewen	Nature of X-ray-induced lesions leading to chromosome aberrations
W. L. Russell	Mammalian Genetics Program
Developmental Biology	
John Papaconstantinou	The regulation of ribosomal RNA synthesis and ribosomal assembly in lens cell differentiation
Mammalian Studies	
R. L. Tyndall	Some ramifications of murine leukemia virus infection *"in vitro"*
H. F. Walburg, Jr.	Influence of microbial flora on repopulation of hemopoietic organs and leukemia induction in irradiated mice
Plant Physiology and Photosynthesis	
A. H. Haber	Exploiting plants for studies of radiation lethality, senescence, and tumor inducibility
Concluding Remarks	
R. F. Kimball	

BONE MARROW CONFERENCES

Since 1957 the Biology Division has cooperated with investigators from many institutions throughout the world in arranging conferences on bone marrow transplantation and chemical protection.

Abstracts of reports presented at each conference are published in *Experimental Hematology*, a journal edited in the Biology Division by C. C Congdon and the Editorial Office, with the assistance of the Technical Publications Department of the ORNL Technical Information Division. Invited papers on selected subjects pertinent to a particular meeting and contributed papers, not connected with the bone marrow conferences but in the field of hematology, are also published in the journal. In 1968 three issues of *Experimental Hematology* (Nos. 15,[1] 16, and 17) were published.

Members of the Division took an active part in the three bone marrow meetings held in 1968:

[1] *Experimental Hematology* No. 15 contains abstracts from the 10th Anniversary Bone Marrow Conference, held November 17–18, 1967, in Oak Ridge, Tennessee.

April 17, Atlantic City, New Jersey

Conference on Bone Marrow Transplantation and Chemical Protection

Chairman: N. B. Everett, University of Washington, Seattle.

Division Reports: Morphologic basis for liver enlargement (W. H. McARTHUR[2,3]); Early proliferation of transplanted spleen colony-forming cells (A. L. KRETCHMAR[3]).

Proceedings: Covered in *Experimental Hematology* No. 16. One contributed paper is included in this issue: The mouse kidney and erythropoietin production (John Kateley, Zoology Department, University of Tennessee). Also included are abstracts from the **Symposium on Myeloproliferative Disorders of Animals and Man**, held May 20–23 in Richland, Washington. Sponsored by the U.S. Atomic Energy Commission and Battelle Memorial Institute–Pacific Northwest Laboratory, the meeting included one paper by a member of the Division: Comparative observations on radiation-induced myeloproliferative disorders in animals and man (A. C. UPTON). Full papers will be published in book form by the Excerpta Medica Foundation.

June 26–28, Padova, Italy

Second International Conference on Germinal Centers of Lymphatic Tissue

Chairman: L. Fiore-Donati, Institute of Pathologic Anatomy, University of Padova, Italy.

Division Reports: Localization of ^{125}I-labeled antigen in germinal centers of mouse spleen: Effects of competitive injection of specific or noncrossreacting antigen (M. G. HANNA, JR., M. W. Francis, and L. C. Peters); Radiosensitivity of the antigen-trapping mechanism and its relation to suppression of the immune response (PAUL NETTESHEIM and M. G. Hanna, Jr.); Germinal centers, Hassall's corpuscles, and epithelioid granulation tissue (C. C CONGDON).

Proceedings: Covered in *Experimental Hematology* No. 17. This issue also includes two contributed papers: Blood volume of the mouse (Charles L. Paxson, Jr.,[4] and L. H. Smith), and Effect of treatment with 5-azacytidine (NSC-102,816) in mice bearing leukemia L1210 (S. Vadlamudi and A. Goldin, Microbiological Associates, Inc., and Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Maryland).

December 5–6, New York, New York

Bone Marrow Conference

Chairman: Dr. Arthur W. Rowe, The New York Blood Center, New York, New York.

Proceedings: To be published in *Experimental Hematology* No. 18. The conference emphasized preservation of hemopoietic, peripheral blood, and lymphatic tissue elements. Papers on topics of importance to preservation studies, such as procurement and utilization, were also presented.

INTERNATIONAL SYMPOSIUM ON NUCLEAR PHYSIOLOGY AND DIFFERENTIATION

A **Symposium on Nuclear Physiology and Differentiation**, the eighth in a continuing series of Latin American conferences initiated in 1961 under the leadership of Alexander Hollaender, was held in Belo Horizonte, Brazil, December 1–6. The symposium was jointly arranged by the Universidade Federal de Minas Gerais, the Biology Division of the Oak Ridge National Laboratory, and the University of Texas, with the support of the National Academy of Sciences, the U.S. Atomic Energy Commission, the National Science Foundation, the Ford Foundation, and the Organization of American States.

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[2] Names of those who presented papers are shown in capital letters throughout this section.

[3] Division Consultant.

[4] Oak Ridge Associated Universities summer student trainee from Kansas State College of Pittsburg, Pittsburg, Kansas.

Serving on the organizing committee for the symposium were Alexander Hollaender, O. L. Miller, Jr., Elliot Volkin, and R. C. von Borstel from the Biology Division; M. J. Moses from Duke University; R. P. Wagner from the University of Texas; C. Pavan from the University of São Paulo; J. Moura Gonçalves from the University of São Paulo at Ribeirão Prêto; and W. Beraldo, C. Diniz, and Giorgio Schreiber from the Universidade Federal de Minas Gerais.

The program was divided into the following sessions: Chromosomal activities during meiosis I, Chromosomal activities during meiosis II, Nuclear activities during development, DNA metabolism during development, Genetic redundancy, Gene expression during differentiation, and Nucleic acids in bacteriophage development. Division members who presented papers were O. L. Miller, Jr., R. C. von Borstel, and Elliot Volkin. Dr. Hollaender gave the introductory and concluding remarks.

1969

TENTATIVE PROGRAM: TWENTY-SECOND ANNUAL BIOLOGY RESEARCH CONFERENCE

The 1969 Symposium on **Protein–Nucleic Acid Interaction**, sponsored by the Biology Division, will be held March 31–April 3 at the Riverside Motor Lodge in Gatlinburg, Tennessee. The organizing committee – Elliot Volkin (chairman), W. E. Cohn, K. Bruce Jacobson, J. W. Longworth, Peter Pfuderer, and R. B. Setlow – has announced the following tentative program:

Monday, March 31

Morning Session

Chairman – Erwin Chargaff, Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, New York

Nucleic Acid and Polynucleotide Structure – Robert Langridge, Department of Chemistry, Princeton University, Princeton, New Jersey

DNA Virus Maturation – R. L. Sinsheimer, Division of Biology, California Institute of Technology, Pasadena, California

Afternoon Session

Chairman – E. P. Geiduschek, Department of Biophysics, University of Chicago, Chicago, Illinois

Mechanisms of Transcription During the Development of Bacteriophages – Waclaw Szybalski, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin

Bacteriophage-Specific tRNA's – S. B. Weiss, Argonne Cancer Research Hospital, Chicago, Illinois

Tuesday, April 1

Morning Session

Chairman – G. M. Tener, Department of Biochemistry, University of British Columbia, Vancouver, Canada

tRNA Structure – Friedrich Cramer, Max-Planck-Institut für Experimentelle Medizin, Göttingen, Germany

tRNA-Aminoacyl Synthetase Recognition – P. C. Zamecnik, Massachusetts General Hospital, Boston, Massachusetts

Wednesday, April 2

Morning Session

Chairman – Ernest Borek, Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, New York

The Separation of Isoaccepting Transfer RNA's and the Possible Role of tRNA in Regulation – G. David Novelli, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

Enzymic Modification of Basic Polynucleotide Units – U. Z. Littauer, Department of Biochemistry, The Weizmann Institute of Science, Rehovoth, Israel

Afternoon Session

Chairman – F. J. Bollum, Department of Biochemistry, University of Kentucky Medical Center, Lexington, Kentucky

RNA-Directed RNA Polymerase – Charles Weissmann, Institut für Molekularbiologie, Universität Zürich, Zürich, Switzerland

DNA-Directed RNA Polymerase – Audrey Stevens, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennesee

Thursday, April 3

Morning Session

Chairman – P. H. von Hippel, Institute of Molecular Biology, University of Oregon, Eugene, Oregon

Association Between Macromolecules – D. L. D. Caspar, Children's Cancer Research Foundation, Boston, Massachusetts

Reconstitution of Ribosomes – Masayasu Nomura, Department of Genetics, University of Wisconsin, Madison, Wisconsin

SYMPOSIUM ON GNOTOBIOTIC RESEARCH

The Association for Gnotobiotics will hold its annual meeting in Oak Ridge June 10–13 at the Alexander Motor Inn. Titled **Symposium on Gnotobiotic Research: Its Importance to Basic Mammalian Research and Human Medicine,** the conference is being planned by D. M. Robie, organizing chairman, and H. E. Walburg, Jr., program chairman.

The primary purpose of the meeting will be to analyze critically the current direction of gnotobiotic research, but its organizers hope to include investigators who are not directly engaged in such research to discuss the relevance of gnotobiotics to their work.

Discussion will concern the influence of microbial flora on immunological mechanisms, on carcinogenesis, on physiology of the cecum, and on nutrition. A new area of interest likely to receive attention is patient care in a sterile environment, especially for patients with leukemia and burns. About 150 participating scientists are expected.

FOURTH CONFERENCE ON BLOOD PLATELETS

Tentative plans are being made for the **Fourth Conference on Blood Platelets** to be held in Oak Ridge the last week of June 1969. Supported by the Biology Division, Oak Ridge National Laboratory, these biennial conferences provide an opportunity for investigators to discuss work on the functions and characteristics of blood platelets.

T. T. Odell, Jr., is organizing the meeting. Previous conferences in the series were held in 1962, 1964, and 1967.

INTERNATIONAL SYMPOSIUM ON THE FERTILITY OF THE SEA

Plans are being made for the ninth Latin American Conference, a **Symposium on the Fertility of the Sea**, to be held December 1–6, 1969, at the University of São Paulo, Brazil. Sponsored by the Oceanographic Institute of the University with the support of the São Paulo State Research Foundation and National Research Council, the conference is being planned in cooperation with Duke University and the Biology Division.

The purpose of the symposium is to assess physical, chemical, and geological oceanographic processes that bear on the fertility and enrichment of the sea. The biological consequences of the different processes that bring about an enrichment of the marine environment will be evaluated and related to ecological and geological patterns of the distribution and abundance of marine organisms. Special attention will be paid to phenomena, processes, and features of the oceans around Latin America.

BONE MARROW CONFERENCES

Several **Bone Marrow Conferences** are projected for 1969. The first will be an evening session on Monday, April 14, 1969, at Atlantic City, New Jersey, in connection with the meetings of the Federation of American Societies for Experimental Biology. Dr. J. P. OKunewick of Allegheny General Hospital will chair the discussion meeting.

The Cooperative Group on Bone Marrow Transplantation in Man plans to hold a work session June 16 and 17, 1969, at Villejuif, France. Two colloquia, one on Transfusion of White Blood Cells and the other on Treatment of Acute Leukemia, will take place June 18–20, 1969, in Paris, France, according to information received from Professor George Mathé of Hôpital Paul-Brousse, Villejuif, France.

Educational Activities

STUDENT TRAINEE PROGRAM

This year 22 juniors from colleges in 17 states were selected on a competitive basis from applicants throughout the United States to serve on temporary summer appointments in the Biology Division. This Summer Student Trainee Program is administered for the United States Atomic Energy Commission by Oak Ridge Associated Universities and is carried out in cooperation with the Oak Ridge National Laboratory.

Under this program, each student is assigned to a specific project which he carries out with the advice and guidance of a senior staff member. At the end of the summer he writes up the results of his work and presents a seminar before all of the Division personnel.

The 1968 students, their affiliations, and the sections to which they were assigned were:

Name	School	Group
Bruce M. Benjamin	Michigan State University East Lansing, Michigan	Nucleic Acid Enzymology
Patricia M. Bihl	St. Mary of the Woods College St. Mary of the Woods, Indiana	Enzymology
Dianne T. Boldin	Barry College Miami, Florida	Cytology and Genetics
Earl A. Burch, Jr.	Augusta College Augusta, Georgia	Pathology and Physiology
Melvin L. Clayton	North Carolina College at Durham Durham, North Carolina	Mammalian Recovery
John W. Cross, Jr.	Vanderbilt University Nashville, Tennessee	Nucleic Acid Enzymology
George L. Higgins III	Colby College Waterville, Maine	Enzymology

Name	School	Group
Margaret J. Howarth	Georgian Court College Lakewood, New Jersey	Yeast, Hymenoptera, and Phage Genetics
Anne H. Jones	Randolph-Macon Woman's College Lynchburg, Virginia	Pathology and Physiology
Carol L. MacGregor	Stetson University De Land, Florida	Radiation Immunology
Kenneth L. Malas	Juniata College Huntingdon, Pennsylvania	Biochemistry of Cell Differentiation
Clifford R. Merchant	Western Maryland College Westminster, Maryland	Nucleic Acid Chemistry
Carol A. Opple	Youngstown State University Youngstown, Ohio	Cell Physiology
John R. Palisano	The University of Tennessee Knoxville, Tennessee	Cell Growth and Differentiation
Betty J. Persons	McPherson College McPherson, Kansas	Cell Growth and Differentiation
Barbara E. Rothstein	Vassar College Poughkeepsie, New York	Plant Physiology and Photosynthesis
Karen M. Royal	College of St. Mary Omaha, Nebraska	Cytology and Genetics
Janice R. Thompson	Eastern Illinois University Charleston, Illinois	Yeast, Hymenoptera, and Phage Genetics
Allen L. Thunberg	North Dakota State University Fargo, North Dakota	General Physiology
Kathy S. Williams	Stetson University De Land, Florida	Radiation Microbiology and Microbial Genetics
Pamela E. Wilton	Westhampton College of the University of Richmond Richmond, Virginia	Enzymology
Kathryn J. Woodle	Oberlin College Oberlin, Ohio	Experimental Hematology

OTHER STUDENTS

The following ten undergraduate students also worked in the Division on temporary summer appointments in 1968.

Name	School	Group
William Hopkins Britt	Tennessee Technological University Cookeville, Tennessee	Administrative Office
Johnny Eugene Burris	King College Bristol, Tennessee	Experimental Animal Facility
Bonnie Kaye Ferrill	Tennessee Technological University Cookeville, Tennessee	Administrative Office
Guy Johnson III	Pennsylvania State University University Park, Pennsylvania	Cell Physiology
G. F. Owens	Cumberland College Williamsburg, Kentucky	Experimental Animal Facility
John Aubrey Rock	Louisiana State University Baton Rouge, Louisiana	Rhynchosciara Genetics

Name	School	Group
Katherine Settles	Knoxville College Knoxville, Tennessee	Media Kitchen
Harry J. Spencer	Knoxville College Knoxville, Tennessee	Nucleic Acid Enzymology
Paula J. Thompson	University of Wisconsin Madison, Wisconsin	Plant Physiology and Photosynthesis
Wanda B. Williams	Tennessee A & I State University Nashville, Tennessee	Administrative Office

SENIOR TRAINEES

Five graduate students were accepted for summer employment to carry out independent research projects in the Division. Listed below are their names, the graduate schools they will attend, and the groups with which they were associated.

Name	School	Group
Richard Albert Dolbeer	University of Tennessee Knoxville, Tennessee	Nucleic Acid Enzymology
William David Hankins	University of Tennessee Knoxville, Tennessee	Biochemistry of Cell Differentiation
Daniel Richard Holladay	Auburn University Auburn, Alabama	Enzymology
Paul Bruce Selby	UT–Oak Ridge Graduate School of Biomedical Sciences Oak Ridge, Tennessee	Mammalian Genetics
Thomas Gordon Wilson	Clemson University Clemson, South Carolina	Enzymology, and Drosophila Cytology and Cell Genetics

THE UNIVERSITY OF TENNESSEE–OAK RIDGE GRADUATE SCHOOL OF BIOMEDICAL SCIENCES

The Graduate School of Biomedical Sciences began its second academic year in September 1968 and now has an enrollment of 16 students, as follows:

J. C. Bagshaw	Sarah A. Goodman
H. Bank	Dale E. Graham
R. W. Barton	A. D. Pavlista
J. F. Calvino	G. B. Price
Ann E. Campbell	K. J. Roozen
E. T. Chin	P. B. Selby
G. Dunn	D. H. Sheehan
S. A. Fairfield	G. Van Denbos

Of the five advanced students engaged in doctoral research, one has been formally admitted to candidacy, and others are scheduled for the near future. Besides the full-time student group, 12 students from other University of Tennessee departments are enrolled for particular courses.

John S. Cook was appointed Associate Director and will assist Director R. C. Fuller in the academic development of the school. W. S. Riggsby was appointed Assistant Professor and will serve until July 1, when he will join the faculty of the Microbiology Department at the University of Tennessee. D. E. Olins continues as Assistant Professor. This full-time staff is supplemented by 66 members of the Biology Division, who teach on a part-time basis.

Candidates for another full-time faculty position are currently being interviewed. R. M. Smillie of the C.S.I.R.O. Plant Physiology Unit and the University of Sydney (Australia) concluded a six-month appointment as Visiting Professor in December.

A five-year training grant of $445,500 from the National Institute of General Medical Sciences, National Institutes of Health, was awarded to the school and is being used to support seven students. Dr. Olins received an NIH Career Development Award, and the following students were awarded national competitive fellowships:

Joseph C. Bagshaw	NSF Predoctoral Fellowship
Paul B. Selby	NSF Predoctoral Fellowship
Gerald B. Price, Jr.	NIH Predoctoral Fellowship
Kenneth J. Roozen	NIH Predoctoral Fellowship
Dale E. Graham	AEC-ORAU Predoctoral Fellowship
Stephen A. Fairfield	AEC-ORAU Predoctoral Fellowship

Of the remaining students, one is supported by an NSF traineeship, one by an NDEA Title IV Fellowship, and one by a Research Assistantship at the Biology Division.

The school continues to operate in its temporary quarters within the Biology Division, but plans for a separate facility are nearing completion. Work on the Student Social Center in Oak Ridge is progressing rapidly, and should be completed by spring.

As part of an accelerated recruiting program, staff members traveled to campuses in the West, Southeast, and Northeast to talk to qualified students about the new school. Visits to campuses in other sections of the country are planned for the coming year.

Cooperative activities with the Biology Division during the past year have included: Advanced Seminar on the Application of Electron Microscopy to Molecular Biology, conducted during October and supported by the National Science Foundation, and the Special Seminar Series, which brings distinguished speakers to the laboratory under the joint auspices of the Division and the Biomedical School. Visitors lecturing under this program and their topics are listed in the LECTURES section, p. 43.

From the inception of the school to the end of the fall quarter of 1968, members of the Biology Division shared faculty have been in charge of the following courses:

Biochemistry	F. J. Finamore
Biophysics	R. B. Setlow
Cell Biology	O. L. Miller, Jr.
Developmental Biology	John Papaconstantinou and Dorothy M. Skinner
Genetics (transmission)	H. I. Adler
Genetics (molecular)	W. E. Barnett
Special Topics in Biomedical Sciences	
Enzyme Kinetics	K. Bruce Jacobson
Special Topics in Biochemistry	F. J. Finamore
Cell Replication and Its Control	A. C. Upton (with J. S. Cook)
Statistics in Biology	M. A. Kastenbaum
Crystallography	J. R. Einstein
Seminar Courses	
Biochemistry	G. David Novelli
Developmental Biology	Tuneo Yamada
Genetics	Roy Curtiss III
Immunology	Takashi Makinodan

Students have been enrolled for Doctoral Research and Dissertation in the laboratories of W. E. Barnett, Takashi Makinodan, G. David Novelli, W. L. Russell, Dorothy M. Skinner, and Elliot Volkin.

Laboratory research rotation assignments have been carried out under the direction of J. F. Albright, W. A. Arnold, W. E. Barnett, R. C. Brown, L. G. Caro, Roy Curtiss III, J. R. Einstein, F. J. Finamore, W. D. Fisher, M. G. Hanna, Jr., K. Bruce Jacobson, F. T. Kenney, C. G. Mead, O. L. Miller, Jr., Audrey S. Niyogi, John Papaconstantinou, R. M. Pearlstein, R. A. Popp, Jane K. Setlow, Dorothy M. Skinner, M. P. Stulberg, R. W. Tennant, Elliot Volkin, and Tuneo Yamada.

In addition, the following members of the shared faculty have lectured in one or more courses: J. G. Brewen, W. E. Cohn, C. C Congdon, D. G. Doherty, J. E. Donnellan, Jr., J. N. Dumont, D. E. Foard, A. H. Haber, J. W. Longworth, Ellen Mattingly, Peter Mazur, E. F. Oakberg, T. T. Odell, Jr., R. O. Rahn, M. L. Randolph, A. C. Upton, H. E. Walburg, Jr., R. A. Wallace, and W. D. Wicks.

ADVANCED SEMINAR ON THE APPLICATION OF ELECTRON MICROSCOPY TO MOLECULAR BIOLOGY

An advanced seminar on the Application of Electron Microscopy to Molecular Biology was given at the Division September 30–October 19. Organized by Lucien G. Caro and sponsored by the Biology Division, the University of Tennessee–Oak Ridge Graduate School of Biomedical Sciences, and the National Science Foundation, this course was designed to teach the operation of the instrument as well as various methods of specimen preparation, with strong emphasis on quantitative techniques. The teaching staff included: Lucien G. Caro, Ann B. Jacobson, and O. L. Miller, Jr., of the Biology Division; Edouard Kellenberger of the Institute of Molecular Biology, Geneva, Switzerland; and Fred Eiserling of the University of California at Los Angeles. In addition, several lectures on special topics were given by guest speakers. The 18 students attending were at the postdoctoral or advanced Ph.D. level and had strong backgrounds in molecular biology.

COURSES TAUGHT

UT–ORNL–Ford Foundation Program. – Under the University of Tennessee–Oak Ridge National Laboratory Cooperative Research Program, initiated by funds from the Ford Foundation, C. C Congdon taught courses in Comparative Animal Pathology during the winter and spring quarters at the University; and A. H. Haber, during the spring quarter, gave a course entitled Advanced Topics in Plant Physiology.

UT–Oak Ridge Resident Graduate Program. – Over the past year five members of the Biology Division taught in the Oak Ridge Resident Graduate Program, which is sponsored jointly by the University of Tennessee and Oak Ridge Associated Universities, Inc. (ORAU). They were: R. W. Tennant (Animal Virology), G. L. Whitson (Concepts in General Zoology), W. D. Gude (Histology), J. D. Regan (Human Genetics), and W. E. Barnett (Seminar in Molecular Biology).

Other Courses. – Marilyn Kerr and Ellen Mattingly taught Embryology and Cell Biology, respectively, at Knoxville College during the fall. Sohan Modak gave the Embryology course during first semester 1967–68, and began it this fall, turning it over to Dr. Kerr late in September. Dr. Mattingly also taught a course sponsored by the National Science Foundation (Modern Biology for High School Teachers) during the second semester (1968).

E. H. Y. Chu offered a four-hour graduate course in Radiation Cytology during the winter quarter at the University of Tennessee, and an advanced genetics seminar (Genetic Basis of Somatic Variation) in the spring.

C. M. Steinberg assisted in teaching a course on Modern Topics in Biology given at the University of Puerto Rico during the spring.

J. D. Regan, for the third straight year, taught a Human Genetics course at the University of Tennessee. This course is offered in the spring through the University's Department of Zoology.

LECTURES GIVEN IN COURSES, INSTITUTES, AND SPECIAL PROGRAMS

W. E. Barnett, during the spring, gave a series of six lectures on the "Molecular biology of nucleic acids" to students in a Modern Topics in Biology course at the University of Puerto Rico.

As part of his travel in the Far East, E. H. Y. Chu lectured on the "Quantitative analysis of mammalian cells" in the Summer Science Seminar of the Academia Sinica, Chinese National Research Council, held in Taipei, Taiwan.

E. B. Darden, Jr., assisted by M. C. Jernigan, gave the first of a series of Public Awareness Seminars at the Oak Ridge Regional Science Experience Center on November 26. Dr. Darden spoke on "Methods in mammalian radiobiological studies," and demonstrated some of the equipment used in these studies.

On August 12, F. J. de Serres spoke on the NASA biosatellite radiation experiments as a participant in the NASA Bio-Space Technology Training Program held at Wallops Island, Virginia.

J. L. Epler, W. D. Fisher, W. D. Gude, and Mayo Uziel participated August 30–September 8 as guest lecturers in a special course on Macromolecular Separations held at the Institute for Scientific Investigation, Caracas, Venezuela.

Rhoda F. Grell spoke on "Meiotic chromosome behavior" in a special course offered to a graduate class in Physiological Genetics at Case Western Reserve University, Cleveland, Ohio, in March.

W. D. Gude lectured in a number of special courses sponsored by ORAU and given in Oak Ridge. His topic was "Autoradiography." Dr. Gude was also active in the Visiting Scientist Program of the Tennessee Academy of Science, giving the following talks at Tennessee high schools:

Topic	School
Careers in laboratory technology	Powell High School, Powell
	Doyle High School, Knoxville
	Brainerd High School, Chattanooga
Biological effects of radiation on mammals	Powell High School, Powell
	Doyle High School, Knoxville
	Brainerd High School, Chattanooga

F. T. Kenney presented a lecture at the University of Missouri Medical School entitled "Regulation of enzyme synthesis and degradation in mammalian tissues." This talk was given as part of the school's Special Topics in Biochemistry Lecture Series.

As part of the cooperative graduate and postdoctoral teaching program between the University of Georgia and the Biology Division, G. David Novelli presented a seminar to the Biochemistry Department of the University on "Studies of transfer RNA."

E. F. Oakberg, W. L. Russell, and A. C. Upton, in July, lectured in the Radiation Biology course for college teachers of biology held at the University of Tennessee College of Medicine, Memphis. Dr. Oakberg spoke on "Radiation effects on the gonads," Dr. Russell on "Radiation genetics," and Dr. Upton on "Radiation and carcinogenesis."

T. T. Odell, Jr., discussed several aspects of his work on the "Steady-state renewal of cells" at the second Public Awareness Seminar held at the Oak Ridge Regional Science Experience Center on December 16.

In February, John Papaconstantinou spoke on "Molecular aspects of lens cell differentiation" to the biology students of Melbourne High School, Melbourne, Florida.

Liane B. Russell was in Cleveland, Ohio, February 27–29, to give two lectures in a special graduate course on Physiological Genetics at Case Western Reserve University. Her topics were "Activity of the mammalian X chromosome" and "Use of sex-chromosome aberrations in mutagenic studies."

W. L. Russell spoke at a joint colloquium of the Zoology, Genetics, and Radiology courses at the University of Wisconsin, October 2–6.

A. C. Upton participated as a guest lecturer in a summer lecture series given at the Anthony J. Lang Laboratories as part of a Summer Research and Teaching Institute in Environmental Medicine, held August 11–17 under the auspices of New York University.

Lecturing in G. L. Whitson's course, Concepts in General Zoology, were W. E. Barnett, M. A Bender, W. L. Carrier, W. D. Fisher, D. E. Foard, M. G. Hanna, Jr., and R. A. Popp.

W. D. Wicks, in early December, gave a series of six lectures on "Biochemical regulation in animal tissues" and directed laboratory sessions in an advanced biochemistry course at the University of Pernambuco, Recife, Brazil.

SPECIAL COURSES TAKEN

J. O. Brick attended a short course on Diseases of Laboratory Animals given March 6–8 at the Ohio State University College of Veterinary Medicine, Columbus, Ohio.

K. R. Layman was in Washington, D.C., in early December for a laboratory training course given at the National Cancer Institute and Microbiological Associates, Inc.

R. O. Rahn attended the NATO summer school on Photochemistry at Menton, France, July 1–24.

J. D. Regan, in late July and early August, attended a Medical Genetics course at the Roscoe B. Jackson Laboratory, Bar Harbor, Maine.

Division Members Working in Other Laboratories

TO CONTINUE EDUCATION

J. G. Farrelly, who continued his studies and research for the Ph.D. degree in biochemistry at the University of Tennessee during 1968, returned to the Laboratory November 18 on a Biology Division Postdoctoral Investigatorship to work in the Enzymology Group with Dr. M. P. Stulberg.

M. L. Davis is continuing his work on a Ph.D. degree in radiation biology at the University of Tennessee under a U.S. Public Health Service Predoctoral Fellowship. Mr. Davis is on leave of absence from the Pathology and Physiology Section. His thesis research is being carried out in the Division's Mammalian Recovery Section under the direction of C. C Congdon.

R. T. Jones is on leave of absence from his position in the Pathology and Physiology Section to continue work on a Ph.D. degree in bacteriology at the University of Tennessee under a U.S. Public Health Service Predoctoral Fellowship. His thesis research will be carried out in the Division's Radiation Microbiology and Microbial Genetics Section under the direction of Roy Curtiss III.

AT THE ATOMIC ENERGY COMMISSION

G. E. Stapleton spent a third year with the Division of Biology and Medicine of the Atomic Energy Commission, Washington, D.C., while on leave of absence from the Biology Division.

1. Cytology and Genetics

R. F. Kimball[a]

Effects of Radiation on Paramecium (1.1–1.4)

R. F. Kimball[a]
G. L. Whitson[b]
E. G. Bailiff[a]
A. A. Francis[a]
Stella W. Perdue
Karen M. Royal[c]

Rhynchosciara Cytology (1.11)

Ellen Mattingly
Crodowaldo Pavan[b]
Shirley P. Ogle
C. L. Parker
Ronald Eckhardt[e]

Molecular Photobiology of DNA (1.5–1.10)

Jane K. Setlow
B. E. B. Moseley[d]
M. E. Boling
K. L. Beattie
Alice A. Mattingly
Dianne T. Boldin[c]
John A. Rock[c]

Chromosome Chemistry (1.12)

D. E. Olins[a,f]
Ada L. Olins[b]

[a]Dual Assignments
[b]Consultant
[c]Student Trainee
[d]Visiting Investigator from Abroad
[e]NASA Predoctoral Fellow
[f]NIGMS Career Development Award

1.1 LOSS OF PHOTOREVERSIBILITY OF MUTATION IN PARAMECIUM

R. F. Kimball E. G. Bailiff
Stella W. Perdue

In a previous report[1] it was shown that 300,000 ergs/mm^2 of light (BL) from a G.E. black light was sufficient or more than sufficient to produce maximum photoreversal of mutation in *Paramecium aurelia* over the whole dose range of light from a germicidal lamp (uv).

Other evidence suggested that for uv, DNA synthesis (*S*) is the end point for conversion of reversible premutational damage to final mutation, just as it is for other mutagens. Thus it seemed probable that photoreversibility would be lost at but not before DNA synthesis. On the contrary, however, it was found that photoreversibility decreased continuously as the time between uv irradiation and BL irradiation was increased and that all photoreversibility is lost by 4 hr after irradiation, whereas no DNA synthesis occurs until after this time because of delay in synthesis by the initial uv exposure.

Two alternative explanations exist: (1) The photoreversible premutation lesions are converted to intermediate, nonphotoreversible forms that are not fixed as mutation until DNA synthesis. (2) The cell loses the ability to photoreverse premutational lesions even though the lesions themselves might remain photoreversible. To distinguish between these alternatives, five groups of cells were irradiated with uv at ½ hr after division. Group 1 was kept without further treatment; group 2 was exposed to BL immediately after uv; group 3 was exposed to BL at 5 hr after division (4.5 after uv); group 4 was exposed to a second dose of uv at 5 hr after division; and group 5 was exposed to a second dose of uv followed immediately by BL.

The results are shown in Table 1.1.1. The results for groups 1, 2, and 3 confirm the earlier results; uv-induced premutational damage can be photoreversed if the BL exposure is given immediately, not if it is given several hours later. Group 4 shows that a second dose of uv increases the mutation yield and so must have induced premutational damage, but group 5 shows that this newly induced damage is no more photoreversible than is the damage induced several hours previously. This shows that alternative 2, the loss by the cell of the ability to photoreverse lesions whether newly formed or old, is the correct explanation. Consequently, loss of photoreversal does not provide evidence for or against the conversion of premutational lesions to a nonphotoreversible form.

Table 1.1.1. Photoreactivation at ½ and 5 hr After Division

Group	First Treatment, ½ hr After Division		Second Treatment, 5 hr After Division		Mutation Yield ($M \pm$ S.E.)
	Uv[a]	BL[b]	Uv[a]	BL[b]	
1	+	–	–	–	0.53 ± 0.05
2	+	+	–	–	0.14 ± 0.03
3	+	–	–	+	0.49 ± 0.05
4	+	–	+	–	1.37 ± 0.07
5	+	–	+	+	1.43 ± 0.08

[a] 1000 ergs/mm^2 of 2537-A uv.
[b] 3000,000 ergs/mm^2 of "black light."

Reference

[1] R. F. Kimball and E. G. Bailiff, *Biol. Div. Ann. Progr. Rept. Dec. 31, 1967,* ORNL-4280, pp. 81–82.

1.2 ISOLATION OF DNA FROM MITOCHONDRIA IN *TETRAHYMENA*

G. L. Whitson W. D. Fisher[1] A. A. Francis

It has been shown that DNA from *Tetrahymena* has a high A + T content (75%), and CsCl centrifugation studies of DNA from whole cells showed two peaks of DNA, one with a buoyant density of $p = 1.688$ and the other (a smaller peak) with a density of $p = 1.680$.[2] Isolation of mitochondrial DNA from *Tetrahymena pyriformis,* strain GL, indicated that it had a buoyant density of $p = 1.684$, and a partial characterization of this DNA indicated that it was probably double-stranded native DNA. We have also isolated whole-cell DNA from *T. pyriformis,* GL-C, and preliminary observations indicate that there are two peaks of DNA, one large peak with a buoyant density of about $p = 1.680$ and a smaller peak of about $p = 1.672$. We have also isolated mitochondria by sucrose gradient zonal centrifugation, but as yet we have not obtained a large enough quantity for DNA isolation and density determinations. Our continuing interest in this problem is based on the following points: (1) Previous studies of buoyant densities of different DNA's show that DNA's with high A + T contents have low buoyant densities (poly d-AT, $p = 1.679$),[3] indicating the possibility that mitochondrial DNA from *Tetrahymena* may be of this type. (2) Recent reports are conflicting concerning both the density and configuration of this DNA in *Tetrahymena.*

References

[1] General Physiology section.
[2] Y. Suyama and J. R. Preer, *Genetics* **52**, 1051 (1965).
[3] C. J. Schildkraut, J. Marmur, and P. Doty, *J. Mol. Biol.* **4**, 430 (1962).

1.3 PHOTOPRODUCTS IN UV-IRRADIATED *EUGLENA* DNA

Karen M. Royal G. L. Whitson W. L. Carrier[1]

Chromatographic isolation of labeled photoproducts (pyrimidine dimers) in acid hydrolysates of organisms has been repeatedly shown to be dependent upon labeling DNA's with exogenous pyrimidine precursors. A severe technical problem was encountered with *Euglena,* since it had been shown in many different laboratories that this organism fails to incorporate exogenous pyrimidine precursors into its DNA. However, the possibility of identifying photoproducts as dinucleotides or trinucleotides was pursued with *Euglena,* since Setlow *et al.* discovered that nuclease-resistant sequences (trinucleotides) could be chromatographed from irradiated DNA of bacteria.

We have therefore subjected isolated *Euglena* DNA, both irradiated and nonirradiated DNA, to enzymatic hydrolysis with snake venom phosphodiesterase. Due to the presence of ^{32}P-containing contaminants in our *Euglena* DNA, we have not yet been able to obtain clean chromatograms, but there is some evidence that nuclease-resistant sequences are present only in the uv-irradiated DNA. Enzymatic hydrolysis of control samples indicates that excess amounts of snake venom phosphodiesterase result in the reduction of nonirradiated *Euglena* DNA to 5′-mononucleotides. Further

investigations concerning this problem are being carried out, particularly with reference to the inactivation of chloroplasts in *Euglena* with ultraviolet light.

Reference

[1] Biophysics section.

1.4 UV-INDUCED PYRIMIDINE DIMERS IN *TETRAHYMENA*. III. RELATIONSHIP TO SURVIVAL

G. L. Whitson A. A. Francis

We reported earlier[1,2] on the excision (I) and photoreactivation (II) of pyrimidine dimers in *Tetrahymena*. This report concerns a quantitative comparison of both the in vivo and in vitro production of thymine-containing pyrimidine dimers in DNA with survival and growth after irradiation at various wavelengths of ultraviolet light has been made. The action spectrum of survival determined by following the colonizing ability of single-cell isolates has a minimum at 2800 A, this wavelength being twice as effective in killing as 2650 A. The uv induction of pyrimidine dimers in vivo in *Tetrahymena* DNA has been found to have no such wavelength dependence, the same number of dimers forming at 2650 and 2800 A with the same initial dose. In contrast to the in vivo finding, the formation of pyrimidine dimers in a solution of DNA isolated from *Tetrahymena* has been found to show strong wavelength dependence, with about twice the formation of dimers at 2650 A than at 2800 A. The differences in the action spectrum of dimer formation in vivo and in vitro are probably due to cytoplasmic shielding of the DNA in cells. Nevertheless, the results indicate that at 2800 A there is at least one unknown uv-induced lesion in addition to pyrimidine dimers in DNA which becomes lethal in *Tetrahymena*.

References

[1] G. L. Whitson, A. A. Francis, and W. L. Carrier, *Biol. Div. Ann. Progr. Rept. Dec. 31, 1967*, ORNL-4240, p. 84.

[2] G. L. Whitson and A. A. Francis, *Biol. Div. Ann. Progr. Rept. Dec. 31, 1967*, ORNL-4240, pp. 84–85.

1.5 TRANSFORMATION OF UV SENSITIVITY AND RESISTANCE IN *HAEMOPHILUS INFLUENZAE*

Jane K. Setlow Alice A. Mattingly

Purified DNA from uv-sensitive and wild-type *H. influenzae* has been used to transform the different strains to uv sensitivity or resistance. Uv-resistant transformants may be readily selected on the basis of uv survival from uv-sensitive competent cultures which have been exposed to transforming DNA. A final slope on the survival curve for such cultures extrapolates to the origin at a value approximately equivalent to the fraction of the population transformed by a drug marker on the same DNA. When the transforming DNA is omitted, or is homologous except for the drug marker, no such tail appears. Clones from survivors of cultures given uv doses in the tail region of survival curves, unlike those from irradiated cultures exposed to homologous DNA, are uv resistant and are able to repair uv-irradiated phage. The transformation of one strain defective in the ability to excise pyrimidine dimers from its DNA (DB112) by the DNA from another excisionless strain (DB116) produces a strain which is not only relatively uv resistant and able to repair phage DNA but also has an excision mechanism. Since the frequency of transformation of these properties is like that of the drug marker in the DNA, it is probable that at least two cistrons are involved in the excision process and that the excision defects in DB112 and in DB116 are in two different cistrons.

Selection for transformation to uv sensitivity has been done with a replica plating technique. Just as transformation to resistance in all cases is accompanied by ability to repair irradiated phage, transformation to sensitivity is accompanied by disappearance of this property. Transformation of wild type to sensitivity by DNA from the most sensitive mutant does not produce cells which have the same sensitivity to inactivation as the mutant, nor is the sensitivity of uv-irradiated transforming DNA on these cells as recipients as high as that on the donor cells. These results suggest that the mutant contains more than one mutation affecting repair of DNA.

1.6 THE SITE OF REPAIR OF UV-IRRADIATED TRANSFORMING DNA

K. L. Beattie Jane K. Setlow

If repair of uv-irradiated transforming DNA only occurs when this DNA is paired or integrated with the genome of the recipient cell, then we would expect to find specific competition for repair enzymes only in DNA which itself is able to pair or integrate. This idea may be tested with a transformation system in which various types of DNA will enter the cell but only homologous DNA will pair or integrate.

We have investigated the effect of irradiated and unirradiated homologous and nonhomologous DNA on

the survival of irradiated transforming DNA in competent wild-type *Haemophilus influenzae* as well as in uv-sensitive mutants which are defective in repair of uv damage. The presence of nonhomologous DNA in the cell causes an increase in the survival of irradiated transforming DNA, whether or not the nonhomologous DNA has been irradiated. This effect is presumably due to competition for an enzyme which breaks down the transforming DNA before it can be integrated. Unirradiated homologous DNA acts like nonhomologous DNA in causing an increase in survival of irradiated transforming DNA. Specific competition, in which the presence of uv lesions in the competing DNA causes a decrease in survival of uv-irradiated transforming DNA, is only found with homologous DNA and in cells which have a repair system in which pyrimidine dimers are excised from their DNA.

It is concluded that competition for repair enzymes occurs only in the case of homologous DNA containing substrates (mostly pyrimidine dimers), and therefore transforming DNA must be repaired only after pairing or integration.

1.7 HOST CELL MODIFICATION OF BACTERIOPHAGE OF *HAEMOPHILUS INFLUENZAE*

M. E. Boling Jane K. Setlow

Host cell modification is a phenomenon in which viruses passed through certain host strains acquire noninheritable properties. We have discovered this phenomenon in *H. influenzae*, as a decrease in the ability of viruses grown on some strains to form plaques on other strains. Phages grown on all seven of the uv-sensitive mutants isolated in this laboratory are restricted on the wild-type host, but phages grown on any mutant are not restricted on any of the other mutants. Wild-type-grown phage, on the other hand, are not restricted on the mutants. The restriction involves the phage DNA, since when the phage coats are removed by phenol the phage DNA taken up by competent hosts shows the same effect.

The apparent relationship between host cell modification and uv sensitivity is confused by the fact that the mutants were obtained by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG), which has an extraordinary tendency to form mutants of *H. influenzae* with host cell modification. Ten of twenty single colonies picked from NTG-treated cultures were like the uv-sensitive mutants in their ability to grow phages from these mutants, although their uv sensitivity did not differ from that of the wild type. However, phages grown on the uv-sensitive mutants are only partly restricted on wild-type cells which have been transformed to uv sensitivity by DNA from DB112, the most uv-sensitive mutant. Phages grown on these transformed cells are not restricted by any of the strains. On the other hand, when DB112 is transformed to uv resistance with either wild-type DNA or with DB116 DNA, the host cell modification properties of DB112 are mostly retained, in that these cells do not restrict phage grown on the uv-sensitive mutants and phages grown on these cells are partly restricted on wild-type cells. Thus selection for uv sensitivity, either from mutagen-treated cells or from cells transformed to uv sensitivity, may cause a selection for host cell modification properties. Among five cases examined of transformation to uv resistance, there was some decrease in the host cell modification of the recipient cells. It is possible that some gene involving uv sensitivity is linked to a host cell modification gene and that they sometimes transform together. The fact that transformation to uv sensitivity or resistance causes a partial change in restriction or host cell modification suggests that there may be more than one gene involved in these properties.

1.8 RECOMBINATION-DEFECTIVE *HAEMOPHILUS INFLUENZAE*

Jane K. Setlow M. E. Boling

One of the uv-sensitive mutants isolated in this laboratory, DB117, can only be transformed with a very low frequency, although it takes up DNA as well as the wild type. It was postulated that the mutant has a defective recombination system. This hypothesis has been tested by measuring the multiplicity reactivation of uv-irradiated phage in this mutant and in other strains. Multiplicity reactivation is the production of viable phage by multiple infection with phages that are nonviable in single infection. It is considered to result from recombination of undamaged parts of nonviable phage.

The uv sensitivity of *H. influenzae* phage is the same in the case of both DB117 and wild-type hosts, under conditions of infection by single virus particles. Both strains are able to repair damage to viral DNA, presumably because of their ability to excise pyrimidine dimers. However, the results concerning phage sensitivity with multiple infection are different for the two strains, in that the final slope of the inactivation curve for DB117 is hardly different from that of the single-infection curves, whereas the final slope for the wild type is about twice that of the single-infection curve.

It is concluded that (1) *H. influenzae* phage make use of the host cell's recombination system and (2) DB117 does indeed have a defective recombination mechanism.

1.9 LACK OF PHOTOREACTIVABILITY OF ULTRAVIOLET-INDUCED CROSS-LINKS IN DNA

R. W. Barton[1] Jane K. Setlow

If uv-induced cross-links between the two strands of DNA are photoreactivable, as claimed earlier,[2] then pyrimidine dimers across the strands are implicated in cross-linking, since uv-induced pyrimidine dimers have been shown to be the photoreactivable uv lesions. However, the earlier result was later shown to be based on faulty reasoning,[3] so that it was not possible on the basis of the available data to say whether cross-links were or were not photoreactivable. Using a new method, we have obtained evidence that cross-links in purified transforming DNA are not affected by photoreactivation with yeast photoreactivating enzyme.

The method is based on the fact that cross-links may be detected by their ability to renature denatured DNA rapidly. In transformation of *Haemophilus influenzae* at neutral pH, denatured DNA is mostly excluded from entrance into the cell. At low pH, however, denatured DNA transforms the cells very efficiently, but native DNA is mostly excluded. Thus the relative amount of transformation by heavily uv-irradiated native and denatured DNA at neutral and acid pH may be used to assess the amount of cross-linking in this DNA. Photoreactivation of the irradiated DNA before denaturation monomerizes the pyrimidine dimers. If at the same time the cross-links are eliminated, we expect (1) the number of transformations from native DNA in the neutral transformation system to increase markedly, (2) the transformations from denatured DNA in the neutral system to decrease, and (3) the transformations from denatured DNA in the low-pH system to increase. Since none of these predictions turned out to be correct, we conclude in accordance with the data that cross-links are not photoreactivable and are not cyclobutane dimers between DNA strands.

References

[1] Student at the UT–Oak Ridge Graduate School of Biomedical Sciences.

[2] J. Marmur and L. Grossman, *Proc. Natl. Acad. Sci. U.S.* **47**, 778 (1961).

[3] K. C. Smith, in *Photophysiology*, ed. by Giese, vol. 2, Academic, New York, 1964.

1.10 TRANSFORMATION IN *MICROCOCCUS RADIODURANS*

B. E. B. Moseley Jane K. Setlow

It was of interest to develop a transformation system in *Micrococcus radiodurans* since this microorganism has an extraordinarily efficient mechanism for the repair of radiation damage in its DNA. Transformation makes possible for the first time in *M. radiodurans* the genetics of radiation repair. It is also possible to irradiate the transforming DNA and assess its reactivation in the bacterial host without irradiating the bacterium and its repair system.

We have been able to transform streptomycin-sensitive *M. radiodurans* to streptomycin resistance by incubating it with purified DNA from a streptomycin-resistant mutant. The transformation is greatly enhanced by the presence of a heat-stable factor from a crude extract of wild-type *M. radiodurans.* The same extract also enhances the frequency of transformation in *Haemophilus influenzae.* The extract increases the frequency of transformation in spite of the fact that it does not increase the amount of DNA taken up by the recipient bacteria. In the case of *M. radiodurans,* DNA uptake is considerably reduced. It seems likely, therefore, that the transformation enhancement factor acts at the level of DNA integration rather than that of DNA uptake.

Transformation is unusual in *M. radiodurans* in that a particular "competent stage" of growth is not required. DNA, either native or denatured, seems to enter the cell readily at all growth stages. However, the single-stranded form transforms with low efficiency. The other unusual aspect of transformation in this system is that a considerably higher concentration of transforming DNA is required than in other transformation systems. This may be because *M. radiodurans* has a superior system for excising nonpaired bases in its DNA, so that much of the transforming DNA which is integrated may be eliminated before it has a chance to replicate.

Pyrimidine dimers are induced normally by uv irradiation in DNA from *M. radiodurans.* However, the transforming activity of this DNA appears to be very uv resistant when assayed in wild-type *M. radiodurans.* The survival of this activity cannot be increased by exposing the irradiated DNA to yeast photoreactivating enzyme under conditions which would give extensive monomerization of pyrimidine dimers. It is concluded that the recipient cells repair pyrimidine dimer damage in transforming DNA as efficiently as they do in their own DNA when the bacteria themselves are irradiated.

1.11 NUCLEIC ACID SYNTHESIS DURING LARVAL DEVELOPMENT IN *RHYNCHOSCIARA*

Ellen Mattingly Ronald Eckhardt

While the sciarid fly *Rhynchosciara* resembles the other Sciaridae in many respects, its unusual habit of living throughout its larval cycle in a compact motile mass appears to increase greatly the degree of physiological synchrony seen among members of any one group. This synchronous development, in addition to the large size of the larvae, has made this insect the organism of choice for studies in developmental biology.

The attention of this laboratory has centered on the problem of nucleic acid synthesis during the development of *Rhynchosciara* and the possible relation of variations in such synthetic activities to the hormonal control of development. While much information is available in the literature on insect hormones, virtually no work has previously been published on DNA and RNA synthesis during the development of any insect. The following sections of this report will present data derived from studies in our laboratory on the general nature of these synthetic activities in *Rhynchosciara* larvae. Although the cells of the larval salivary gland in *Rhynchosciara* have received much attention because of their very large polytene chromosomes, other larval tissues also show a high level of polyteny in their chromosomes. Such tissues as the intestine, gastric caeca, Malpighian tubules, and tissues associated with the tracheal system have cells whose polytene chromosomes exceed in size those of the salivary gland cells of *Drosophila.* While these tissues are thought to have widely differing functions in insect development, they have in common the feature that no mitotic divisions are seen after the larvae hatch from the egg, and the homologous chromosomes are always tightly paired. Furthermore, most of these tissues are totally broken down during the period of pupation.

One larval tissue that shows strikingly different behavior during development is the testis. In this tissue, both mitotic and meiotic divisions can sequentially be seen, and development in this tissue results in a functional cell, that is, the sperm. The spermatogonia contain, furthermore, a fairly large chromosome that is not seen in any of the nongonadal tissues and whose function is completely unknown.

With the use of tritium-labeled precursors and high-resolution autoradiography, it has been possible to compile for the first time a complete and well-integrated picture of nucleic acid synthesis during insect larval development. With the data shown in Figs. 1.11.1 and 1.11.2, it is now possible to ask very specific questions about developmental events at the molecular level.

The general pattern of DNA synthesis in the larval polytene tissues of *Rhynchosciara* has recently been published.[1] While this is the only such information that has been obtained for any insect, recent work in other laboratories indicates that similar patterns of synthetic activity will be found in other diptera.

More extensive information has been obtained on the patterns of RNA synthesis in the polytene tissues. It is immediately apparent that RNA synthesis is intimately related to the larval molting cycle, dropping virtually to zero during ecdysis and reaching a peak during mid-instar in the first three periods. The picture in the very long fourth instar is considerably more complex, with peak synthetic activity shortly after the third molt and with remarkable variation in activity subsequently.

It is immediately apparent that RNA synthesis in the salivary glands parallels that of DNA, with some diminution of activity during mid-fourth instar and with a subsequent rise to a very high level during the period immediately prior to the prepupal molt.

In the intestine and Malpighian tubules, after the initially very high synthetic activity, a low level of incorporation is seen constantly up to the period of pupation, when lysis may occur. Although the gastric caeca has been thought by many insect physiologists to function merely as an extension of the intestinal wall, it is obvious that this tissue behaves quite differently from the intestine with respect to RNA synthesis, with a virtually complete cessation of synthetic activity during the latter half of the fourth instar.

Since all of these tissues contain polytene chromosomes, it is theoretically possible to relate this overall picture of RNA synthesis activity to the activity of specific chromosomal loci. While these analyses are still in progress, some particularly interesting data have already been accumulated. The one chromosomal segment that shows obvious variation in activity in the different tissues and also varies during development within the same tissues is that segment of the X chromosome, usually heterochromatic in appearance, that is thought to contain the nucleolar-organizer region.

During the fourth instar, prior to the beginning of cocoon formation, this heterochromatic segment is surrounded by an accumulation of pyroninophilic material that labels intensely with uridine-^{3}H. During the latter half of the fourth instar, in the salivary gland, this aggregate gradually breaks up into spherical bodies that appear to float freely in the nucleoplasm. These

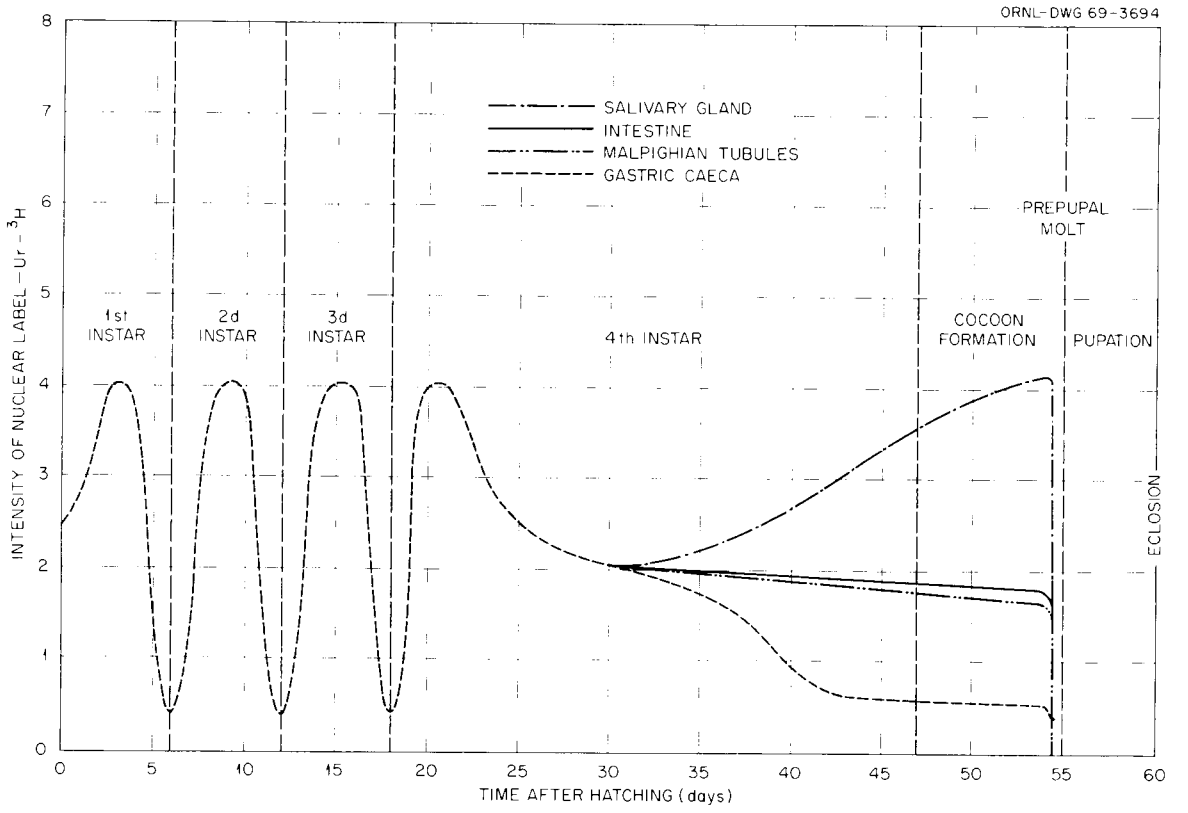

Fig. 1.11.1. Variations in RNA Synthesis During Larval Development in *Rhynchosciara*.

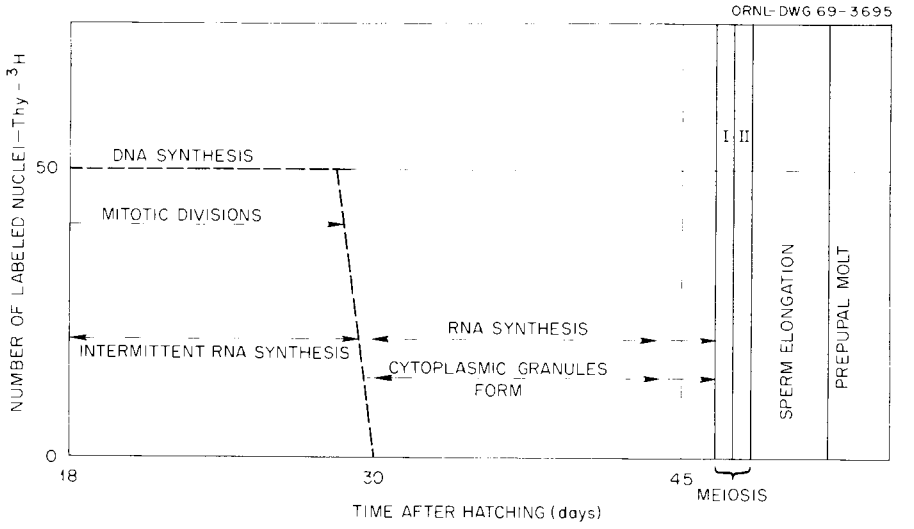

Fig. 1.11.2. Diagrammatic Representation of the Chief Developmental Phenomena Seen in Spermatogenesis in *Rhynchosciara* Testis.

bodies gradually increase in size and number, so that ultimately there are at least 100 such particles detached from the chromosome. Most of these bodies have a very dense core that stains positively with Methyl Green and Feulgen stains, shows an intense green fluorescence with Acridine Orange, and is extracted by treatment with DNAse. In only two other organisms, that is, the extensively studied amphibian oocytes and the cells in the float pad of the Sarcophaga, have such nucleolar bodies been reported.

In the salivary gland these nucleoli label rapidly and intensely with uridine-^{3}H. At the time that cocoon formation starts and the sequence of major puffs is seen on the four chromosomes, all activity ceases at the nucleolus-organizer region. The nucleoli, however, continue to incorporate label and appear to increase in size until the period immediately before the prepupal molt (at 12 hr), at which time the nucleoli cease to incorporate label and gradually disappear. After this cessation of nucleolar label the polytene chromosomes continue to incorporate uridine-^{3}H, and, indeed, at least two large RNA puffs appear subsequently. This suggests the possibility that for a limited period of time, messenger and/or transfer RNA synthesis may continue in the absence of ribosomal RNA synthesis, and sucrose gradient analyses tend to support this hypothesis. Recent experiments show that larvae in which the nucleolar organizer is no longer active can be injected with ecdysterone. In these larvae, after 24 hr an unusually large accumulation of pyroninophilic material is again visible around the nucleolus-organizer, and the disperse nucleoli reach the largest size ever seen in our laboratory. Sucrose-gradient analyses of RNA's extracted from larvae during intervals in the normal course of development have already been prepared in our laboratory. It will be of interest to compare with these the RNA's profiles obtained from such ecdysterone-treated larvae.

In other tissues having polytene chromosomes, there is no evidence of any such disperse nucleoli. The possibility therefore exists that there is specific amplification of certain segments of DNA in the salivary gland to form these disperse nucleoli that does not occur in the other tissues. Experiments are currently in progress to test this hypothesis.

The testis in *Rhynchosciara* has developed into an exceptionally interesting system whose potential is just being recognized. Since very little was known about this system, basic developmental studies were necessary before more sophisticated questions could be asked. Figure 1.11.2 shows diagramatically the data that have been accumulated on the sequence of events in the development of the testis.

During the first two weeks of the fourth instar, smears of the testis show large numbers of dividing cells, and autoradiographs after a 60-min pulse of thymidine-^{3}H show that approximately 50% of the nuclei incorporate label. The DNA synthetic period in the cell is followed by a short and cytologically unusual prophase, with some RNA synthesis taking place at this time.

At approximately mid-fourth instar, the spermatogonia abruptly stop DNA synthesis, and no mitotic divisions are seen subsequently. The spermatogonial chromosomes become somewhat diffuse in appearance, although they remain recognizable as discrete units. This diffuse appearance of the chromosomes is accompanied by a high rate of RNA synthesis. Simultaneously, granules appear in the cytoplasm that appear, in electron micrographs, to be very large spherical mitochondria surrounding dense cores of protein.

Shortly after the beginning of cocoon formation, the spermatogonia pass synchronously through meioses I and II, and sperm elongation also occurs synchronously.

Since comparatively little is known of the fundamental nature of the events occurring during spermiogenesis, this system offers obvious advantages. Preliminary biochemical analyses of DNA and RNA synthesis in this tissue indicate that the testis will indeed be of exceptional value.

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1.12 HISTONE-DNA INTERACTION

D. E. Olins

The basic histone proteins of eukaryotes appear to modify the macromolecular conformation of DNA. The purpose of the present studies is to define the influence of a single class of histones on DNA structure and to determine the site of binding of histone to DNA.

Complexes of lysine-rich histones (f1) with native calf thymus DNA were prepared in soluble form by a salt gradient dialysis method and examined by a variety of physicochemical methods. Absorption spectroscopy and circular dichroism revealed minimal differences between DNA and histone-DNA complexes, suggesting that the DNA remains in the B conformation with histone present. Thermal denaturation and thermal renaturation studies indicated that the presence of

histone resulted in a marked stabilization of DNA structure and permitted rapid renaturation of denatured regions. Maximal renaturation was effected with as few as 20 to 40 histone molecules per DNA molecule (molecular weight, 10^7). Sedimentation and viscosity measurements of the complexes indicated that, at infinite dilution, the particles behave as single DNA molecules with associated histones. Evidence for concentration-dependent aggregation of the histone-DNA particles was also obtained. Dye-binding studies suggested that lysine-rich histone does not bind in the small groove of DNA; the presence of histone on DNA does not inhibit actinomycin binding. Furthermore, evidence was obtained indicating that histone might bind within the large groove of DNA; histone complexes with unglucosylated T2* DNA were less effective substrates for glucosylation by α-glucosyl transferase than was T2* DNA.

The present studies suggest that the DNA of an f1 histone-DNA complex remains in the B form and that the f1 histone molecules bind in the large groove and protect alignment of the DNA strands.

2. Mammalian Cytology and Cell Genetics

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Participation in Cooperative Programs:

AEC-NCI Cocarcinogenesis (30.13, 30.14)

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3. Mammalian Biochemical Genetics

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3.1 PRIMARY STRUCTURE OF THE ALPHA CHAINS OF HEMOGLOBIN FROM C3H MICE

R. A. Popp J. E. Attrill[1]
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We previously reported[2] that the α chains of hemoglobin from C3H mice existed in more than one form. Amino acids valine and glycine, isoleucine and valine, and serine and asparagine were present at positions α25, 62, and 68 respectively. Position 25 is in tryptic peptide αT-4, and positions 62 and 68 are in tryptic peptide αT-9. The fortuitous finding that both α62 and 68 contained two forms of amino acids, both in the same tryptic peptide, provided suitable material to determine whether the heterogeneity of the α chains of C3H hemoglobin results from gene duplication or translational variations of ambiguous codons. The premise is that two forms of αT-9 should be present if the heterogeneity results from gene duplication but four forms should be present if the heterogeneity results from translational variations of ambiguous codons.

The heterogeneous forms of αT-9 could not be separated by chromatography on Dowex 50-X2 or Dowex 1-X2 resins. However, paper chromatography for 40 hr, using butanol:acetic acid:water (4:1:5) as solvent, resolved αT-9 into two chromatographic regions. Peptides from the two regions were eluted from the chromatography paper and hydrolyzed in 6 *N* HCl for 20 hr. Amino acid analyses showed that αT-9 in the faster chromatographic region contained four units of aspartic acid, three units of serine, little or no valine, and one unit of isoleucine.[3] The αT-9 in the slower chromatographic region contained five units of aspartic acid, two units of serine, one unit of valine, and little or no isoleucine.[3] These results established that αT-9 of C3H hemoglobin exists in only two forms; one has isoleucine and serine at positions α62 and 68, respectively, and the other has valine and asparagine (analyzed as aspartic acid on the amino acid analyzer) at positions α62 and 68 respectively. One must conclude from such data that the heterogeneity of α-chain polypeptides in C3H hemoglobin results from gene duplication and not from translational variations of ambiguous codons.

Quantitative analyses of αT-4 and αT-9 also showed that the two forms of α-chain polypeptides appear in unequal amounts. The $\alpha 25^{Val} 62^{Ile} 68^{Ser}$ and the $\alpha 25^{Gly} 62^{Val} 68^{Asn}$ polypeptides constitute 70 and 30%, respectively, of the total α chains found in the hemoglobin from adult C3H mice. Possible mechanisms that regulate the unequal synthesis of these two forms of α chains are under investigation.[4]

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3.2 INHERITANCE OF HEMOGLOBINS IN MICE

R. A. Popp Diana M. Popp

Solubility analyses of mouse hemoglobins in phosphate buffers,[1] supported by analyses of the primary structures of the α-chain polypeptides in mouse hemoglobins,[2] have shown that the α chains of C57BL, NB,

and SEC mice differ from one another. Solubility analyses of hemoglobins from C3H.B and CBA mice[3] indicated that the α chains of these hemoglobins differed from the α chains of the above three strains of mice. Moreover, solubility analyses suggested that the α chains of C3H, SEA, FU, WC, and FLEX mice might be different from the α chains of all the strains of mice mentioned above. This study was done to establish that C3H.B and CBA mice have a unique allele at the *Hba* locus and also that C3H, SEA, FU, WC, and FLEX mice have yet another allele, not previously described, at the *Hba* locus.

Mice of the seven strains were mated to C57BL and SEC mice, and the F_1 hybrids were intercrossed or backcrossed to study the inheritance of these hemoglobin phenotypes among mice. Hemoglobin from each mouse was tested for its solubility and crystalline properties in phosphate buffer to classify each mouse for its hemoglobin genotype. Such genetic studies established that C3H.B and CBA mice have similar α-chain phenotypes and that C3H, SEA, FU, WC, and FLEX mice have similar α-chain phenotypes; moreover, their α-chain phenotypes were indeed different from those previously described for C57BL, NB, and SEC mice.

Chemical analyses showed that the α chains of hemoglobin from C3H.B mice exist in two forms which differ at position α68 only; one form has serine and the other has asparagine at α68 (ref. 4). Quantitative analysis showed that each form represents approximately 50% of total α chain. Similar studies on hemoglobin from C3H mice[5] showed that its α chains exist in two forms which differ at positions α25, 62, and 68 and that the two forms are not present in equal quantities.

Hemoglobins from C3H.B and C3H mice represent the standard prototypes for the fourth and fifth α-chain phenotypes found among laboratory mice. These five α-chain phenotypes were found during an examination of approximately 30 strains of laboratory mice, and they may represent only a fraction of the polymorphic forms of hemoglobins among feral mice. Although there are direct associations between the chemical structures of these hemoglobins and their solubility in phosphate buffer, none of the hemoglobin variants demonstrate a physiological detriment to the mouse, as evidenced by the normal frequencies of the various classes of these hemoglobins which appear among the F_2 and backcross progeny examined.

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3.3 LINKAGE OF DUPLICATE GENES AT THE ALPHA-CHAIN LOCUS IN MICE

R. A. Popp Diana M. Popp

Chemical analyses of the primary structures of the α chains of hemoglobin from C3H mice established that the duplexity of amino acids at positions α25, 62, and 68 results from duplicate genes at the hemoglobin locus rather than translational variations of ambiguous codons.[1] Because the hemoglobin phenotypes segregate as though they are controlled by a single gene or closely linked genes among F_2 and backcross populations of mice, the duplicate genes must be fairly tightly linked.[2,3] This study was done to determine more precisely the degree of linkage between the duplicate genes at the hemoglobin locus in strain SEC mice.

(C57BL × SEC)F_1 mice were backcrossed to mice of both parental strains. The hemoglobins of each parental strain and F_1 mice have very different solubilities in phosphate buffers.[4] Mixtures of hemoglobins with α chains of known chemical formulas showed that recombination of genes at the hemoglobin locus either by independent assortment or by crossing-over between linked genes on chromatids of SEC origin in F_1 mice would result in offspring whose hemoglobin phenotype would have an intermediate solubility in phosphate buffer as compared with hemoglobins from C57BL and (C57BL × SEC)F_1 mice or (C57BL × SEC)F_1 and SEC mice, depending on the backcross progeny being analyzed.

Hemoglobin samples from a total of 551 backcross mice were analyzed; no progeny showed a genetic recombination between the duplicate α-chain genes in SEC mice. Thus the recombination frequency at the 95% confidence limits is not greater than 0.0054. These data are consistent with earlier studies which showed that the α-chain phenotypes of F_2 and backcross progeny of (C57BL × BALB/c)F_1 and (BALB/c × NB)F_1 mice were controlled by single or closely linked genes.[2,3]

The existence of duplicate genes at the α chain of hemoglobin locus *Hba* in the mouse necessitates some means to designate such duplications. Some polypeptides that appear as the only form of α chains in C57BL and NB mice also appear as one of the duplex set of α chains in other strains of mice.[1] The symbols

Table 3.3.1. *Hba* Alleles and α-Chain Structure of Mouse Hemoglobins

Strains of Mice	*Hba* Alleles	Chemical Structures
C57BL/Cum	$Hba^{1\,a}$	$\alpha_2{}^{B\,a}$
BALB/cJ	$Hba^{2,3\,b}$	$\alpha_2{}^{68\,Ser}$; $\alpha_2{}^{68\,Thr}$
NB/RI	Hba^{4}	$\alpha_2{}^{25\,Val}{}_{62\,Ile}{}_{68\,Ser}$
C3H.B/St	$Hba^{1,2}$	$\alpha_2{}^{B}$, $\alpha_2{}^{68\,Ser}$
C3H/Cum	$Hba^{1,4}$	$\alpha_2{}^{B}$, $\alpha_2{}^{25\,Val}{}_{62\,Ile}{}_{68\,Ser}$

[a]Designated as the standard;[5] B is the official abbreviation for strain C57BL.

[b]An abbreviated form is preferable; therefore *Hba* is used to designate the compound locus containing two recognized genetic units or genes, and the superscripts identify the kinds of α-chain polypeptides produced.

suggested to designate known alleles at the *Hba* locus among inbred strains of mice are shown in Table 3.3.1.

References

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3.4 AMINO ACID SEQUENCE IN THE BETA CHAIN OF MOUSE HEMOGLOBINS

R. A. Popp W. S. McEwen

Differences among the tryptic peptides of the β chain of hemoglobins from C57BL and BALB/c mice are correlated with differences in the electrophoretic patterns of these hemoglobins in starch gels.[1] Studies on the sequence of amino acids in the β chain of C57BL hemoglobin have been in progress for over a year. The amino acid sequences for several of the tryptic peptides are reported here.

Tryptic peptides of the β chain were separated by column chromatography over Dowex 50-X2 resin.[2] The sequences of amino acids in the smaller peptides were determined by direct Edman degradation. The larger tryptic peptides were cleaved further by chymotrypsin, pepsin, or papain to produce fragments suitable for Edman degradation. The results are shown in Table 3.4.1.

The β chain of mouse hemoglobin is homologous to the β chain of man and other mammalian hemoglobins.[3] The principal differences between the β chains of man and mouse is that the latter has a larger number of isoleucine and a smaller number of proline residues. Preliminary studies show that the β chains of C57BL and BALB/c hemoglobins differ in at least three β-chain tryptic peptides. The positions of the amino acid replacements in βT-2, βT-3, and βT-14 will be studied

Table 3.4.1. Sequence of Amino Acids in the Beta Chain of C57BL Hemoglobin

Tryptic peptides	βT-1 βT-2
Amino acids	Val His Leu Thr Ala Glu Asp Lys Ser Ala (Val Gly Ala Leu)[a] Try Gly Lys Val Asn Ala Asp Glu
Position numbers	8 17
	βT-3 βT-4
	Val Gly Gly Glu Ala Leu Gly Arg Leu (Leu Val Val Tyr Pro) Try Thr Glu Arg Tyr Phe Ser Ser Phe
	30 40
	βT-5 βT-6 βT-7 βT-8
	(Gly Asp Leu Ser Ser Ala Asp Ala Ile Met Gly Asn Ala) Lys Val Lys Ala His Gly Lys Lys
	59 61 65 66
	βT-9
	Val Ile Thr Ala Phe Ser Asp Gly Leu Asn His Leu Asp Asn Leu Lys (Gly Ser Phe Ala Thr Leu
	82
	βT-10 βT-11
	Ser Glu) (Leu His Cys Asp) Lys Leu His Val Asp Pro Glu Asn Phe Arg (Leu Leu Gly Asn Val Ile
	95 104
	βT-12 βT-13
	Val Ile Gly Leu Met His His Leu Gly) Lys Asp Phe Thr Pro Ala Ala Gln Ala Ala Phe Gln Lys
	120 132
	βT-14 βT-15
	Val Val Ala Gly Val Ala Ala Ala Leu Ala His Lys Tyr His
	144 146

[a]Regions within parentheses have not been established to date.

once the amino acid sequence of the entire β chain of C57BL hemoglobin has been completed. The electrophoretic heterogeneity of the diffuse hemoglobin in BALB/c mice suggests that its β-chain locus may be compound in a manner similar to that described for the β-chain locus of C3H mice.[4]

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3.5 STUDIES ON MURINE ERYTHROCYTE STROMA

Diana M. Popp R. A. Popp
L. H. Elrod[1]

Most fundamental physiological and biochemical processes occur at membrane surfaces in a water medium. Electron photomicrographs have revealed the physical architecture of cell membranes, but their chemistry and chemical organization are not well defined. Our studies on antigen isolation[2] and antibody purification[3] prompted a study on the physicochemical properties of red cell stroma. The membrane of red cells can be collected free from other subcellular structures that are common to most nucleated cells, and erythrocyte membranes should yield more homogeneous preparations than membranes collected from more complex tissues, such as spleen and liver, that contain many cells with specialized functions.

Red cells were collected in a sodium citrate solution and washed in 0.85% NaCl to remove serum. The washed red cells were then lysed in distilled water, and the stroma was pelleted by centrifugation and washed further in 0.1% NaCl until light pink in color. Subsequent washing was done in phosphate-buffered saline until the supernatant became clear.

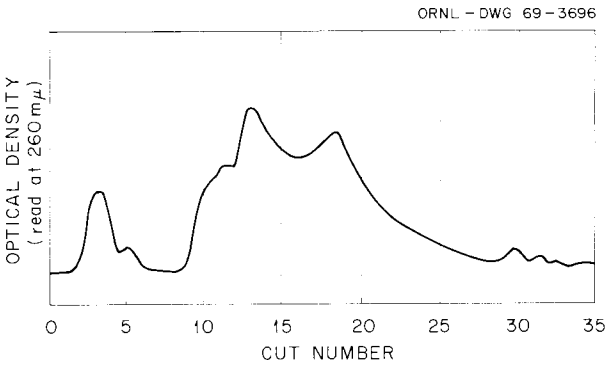

Fig. 3.5.1. Elution Profile of Erythrocyte Stroma in a Sucrose Gradient. Cuts 2 to 7 contain some free hemoglobin; cuts 10 to 16 contain the white or creamy stroma; and cuts 17 to 22 contain the red stroma.

Zonal centrifugation in a sucrose gradient (25–55% sucrose) in a B-XV rotor produced five peaks that were incompletely resolved. The concentration of H-2 antigens among the five peaks was determined by hemagglutination inhibition. The highest concentration of H-2 antigen was found in cuts 14 and 15 (Fig. 3.5.1), which correspond to the lighter, white or creamy, fraction of stroma that is obtained during centrifugation of stroma in phosphate-buffered saline. Such fractions have been prepared from stroma from eight strains of mice, namely, A/Sn, A.CA, C3H, C57BL/6, C57BL/10.D2, C57BL/6.H-12$^{a/a}$, DBA/2, and RFM. Fingerprints of tryptic peptides from each of these show marked similarity, with unique differences among certain strains of mice. Genetic experiments are in progress to determine whether any of the unique peptides can be correlated with various phenotypic markers that are present on red cells from these strains of mice; particular emphasis is being given to H-2 specificities.

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4. Fungal Genetics

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Radiation Mutagenesis (4.1–4.4)

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Chemical Mutagenesis

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Recombination

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Participation in Cooperative Programs:

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AEC-NASA Space Biology (30.54, 30.55)

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[d]Consultant

4.1 COMPUTER PROGRAMMING OF STATISTICAL ANALYSIS OF SPECIFIC LOCUS STUDIES IN *NEUROSPORA CRASSA*

Diana B. Smith[1] F. J. de Serres

A series of computer programs have been written to facilitate the statistical analysis of data obtained in forward-mutation experiments in *Neurospora crassa* at specific loci. The experiments are designed to study the genetic effects of various mutagenic treatments, and assays are made to determine (1) inactivation or killing of conidia and (2) the induction of mutations at two specific loci in the adenine-3 (*ad-3*) region. Since the forward-mutation experiments are performed on a genetically marked two-component heterokaryon of this haploid organism, recoverable mutations in the *ad-3* region consist both of point mutations at the *ad-3A* and *ad-3B* loci as well as chromosome deletions covering each locus individually or both loci simultaneously. The *ad-3* mutations recovered are subjected to a series of genetic tests to determine genotype and to distinguish point mutations from chromosome deletions. In addition, tests for allelic complementation are performed on the *ad-3B* point mutations to obtain a presumptive identification of the genetic alterations at the molecular level.

The computer program entitled *Jug Analysis Program* summarizes the raw data from forward-mutation experiments to provide an analysis of (1) survival of the heterokaryotic fraction of conidia and (2) overall forward-mutation frequencies for recessive lethal mutations in the *ad-3* region. Survival ratios are determined, 95% confidence intervals are computed, and the data are plotted. The forward-mutation frequency obtained with each treatment is determined from the counts of nonmutant and mutant colonies, 95% confidence intervals are computed, and a curve is fitted to the data by means of regression analysis. By using a *Special Plotting Program*, two mutation induction curves can be compared; the program gives a statistical test of the

difference between slopes and then, assuming that the difference is not significant, determines whether the distance between the curves is significant.

The program entitled *Mutant Analysis Program* is used to process the different genetic tests. Test scores on four separate input cards are processed with this program, which lists (1) all test data for each mutant, (2) all mutants having incomplete test data, (3) all mutants with complete test data, and (4) all mutants with inconsistent test data. The program then tallies the number of mutants of each type per treatment and calculates the fraction and forward-mutation frequency of each type. The program also computes a dose power regression for all the different categories of mutants. These results are punched on cards to make it possible to compare (with the *Compare Program*) or combine (with the *Combined Program*) different sets of data. The *Compare Program* computes the same statistics as the *Special Plotting Program.*

The program entitled *Chi Square Program* processes the data on allelic complementation on *ad-3B* point mutations. In these tests mutants are classified as nonpolarized, *ad-3B(R-NP);* polarized, *ad-3B(R-P);* or noncomplementing, *ad-3B(R-NC).* Using the numbers observed the program computes χ^2 for between doses in the same experiment and then (assuming no significant χ^2 within) between experiments.

The programs described were written for an IBM 360, model 75 computer at ORNL.

Reference

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4.2 EFFECT OF PHOTOREACTIVATION ON THE SPECTRUM OF UV-INDUCED MUTATION IN THE *ad-3* REGION OF *NEUROSPORA CRASSA*

F. J. de Serres B. J. Kilbey[1]

Introduction. – We have already shown[2] in an experiment with a haploid wild-type strain that there is but a single type of premutational damage induced by uv that results in point mutations (*ad-3^R*) and that repair of a fraction of this damage with photoreactivation affects all types of mutants similarly. Forward mutations in the *ad-3* region in a two-component heterokaryon consist of both point mutations and chromosome deletions (*ad-3^{IR}*) which result from qualitatively different lesions with x irradiation.[3] In the present experiment with a two-component heterokaryon, we are attempting to study the effect of photoreactivation on uv-induced damage leading to *ad-3^{IR}* mutations. In a previous report[4] on this experiment, we showed the effect of photoreactivation on uv-induced inactivation of heterokaryotic conidia and the overall induction of forward mutations in the *ad-3* region. The DRF (uv exposure in the dark/uv exposure in the light) for both effects was about 0.6. Samples of *ad-3* mutations from each of the exposures in the dark and with photoreactivation were subjected to more detailed genetic analysis (1) to distinguish between *ad-3^R* mutations and *ad-3^{IR}* mutations and (2) to characterize the point mutations at the *ad-3B* locus (*ad-3B^R*) at the molecular level.

Results. – The genetic analysis of the *ad-3* mutations has shown (Fig. 4.2.1) that (1) *ad-3^R* mutations increase as the 2.04 power of the dose in the dark and the 2.02 power of the dose with photoreactivation, (2) *ad-3^{IR}* mutations increase as the 2.60 power of the dose in the dark and the 2.67 power of the dose with photoreactivation, (3) the difference between the slopes of *ad-3^R* mutations and *ad-3^{IR}* mutations is significant (P = 0.03), (4) *ad-3^R* mutations occur about 15 to 20

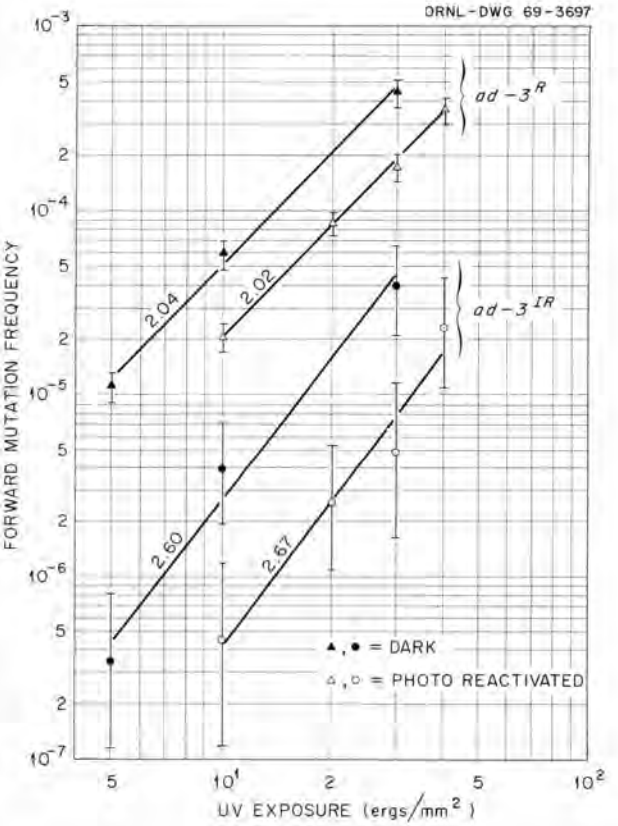

Fig. 4.2.1. Mutation-Induction Curves for *Ad-3^R* and *Ad-3^{IR}* Mutations After Treatment with UV With and Without Photoreactivation.

times more frequently than *ad-3^{IR}* mutations depending on the level of uv exposure, and (5) both classes of mutation show a reduction in frequency with photoreactivation; *ad-3^{R}* mutations have a DRF of about 0.6 and *ad-3^{IR}* mutations have a DRF of about 0.8.

The tests for allelic complementation of the *ad-3B^{R}* mutations showed, in the dark: nonpolarized 37.7%, polarized 12.6%, and noncomplementing 50.3%; with photoreactivation: nonpolarized 38.2%, polarized 11.1%, and noncomplementing 49.3%. The differences between these percentages are not significant (P = 0.83).

Discussion. – The data from the genetic analysis of the *ad-3* mutations show that uv induces both *ad-3^{R}* mutations and *ad-3^{IR}* mutations and that they have different induction kinetics; whereas *ad-3^{R}* mutations increase as the square of the uv exposure, *ad-3^{IR}* mutations increase as the 2.6 power of the uv exposure and may be a mixture of two- and three-hit events. Furthermore, these two classes of mutation show significantly different dose reduction factors following photoreactivation, *ad-3^{IR}* mutations showing a greater effect than *ad-3^{R}* mutations. From these results we have concluded that *ad-3^{R}* and *ad-3^{IR}* mutations result from qualitatively different lesions after uv exposure and that the lesions resulting in *ad-3^{IR}* mutations are repaired preferentially following photoreactivation.

References

[1] Mutagenesis Research Unit, Institute of Animal Genetics, Edinburgh, Scotland.

[2] B. J. Kilbey and F. J. de Serres, *Mutation Res.* **4**, 21–29 (1967).

[3] F. J. de Serres *et al., Brookhaven Symp. Biol.* **20**, 56–76 (1968).

[4] B. J. Kilbey and F. J. de Serres, *Biol. Div. Semiann. Progr. Rept. Jan. 31, 1967,* ORNL-4100, pp. 50–51.

4.3 ENZYMOLOGY OF THE *ad-3* MUTANTS OF *NEUROSPORA*

C. R. Fisher

Although a tremendous amount of information has been accumulated on the *ad-3* mutants of *Neurospora* in various types of genetic analyses, no detailed study of the enzymes controlled by the *ad-3A* and *ad-3B* loci has been undertaken. The present program has been successful in correcting this situation.[1] We have succeeded in producing one of the biochemical intermediates, 5′-phosphoribosyl-5-aminoimidazole-4-(*N*-succinocarboxamide) (SAICAR), by two methods, and a biochemical company has been persuaded to provide the compound on a limited scale commercially. These advances make the assay for the enzyme phosphoribosyl-amino-imidazole-succinocarboxamide synthetase [5′-phosphoribosyl-4-carboxy-5-aminoimidazole: L-aspartate ligase (ADP) EC 6.3.2.6] feasible as a routine procedure. The enzyme assay is based upon the reversibility of the reaction: ATP + 5′-phosphoribosyl-5-aminoimidazole carboxylate (CAIR) + aspartate $\overset{Mg^{2+}}{\rightleftharpoons}$ SAICAR + ADP + P_i. Synthetase from *N. crassa* has been purified 100-fold by ammonium sulfate fractionation, hydroxylapatite chromatography, and DEAE chromatography. Mg^{2+}, ADP, and phosphate or arsenate are required for the reverse reaction. Aspartate and ATP, which are utilized in the forward reaction, inhibit the reverse reaction. The divalent cations Mn^{2+} and Co^{2+} can partially replace Mg^{2+} as activators. The optimum pH is 6.0, and the reaction rate is greater at 37° than at 25 or 30°. The K_m for the reverse reaction is 1.2 to 1.3 × 10^{-4} *M*. Soluble protein preparations from *ad-3B* mutants contain synthetase activity, whereas preparations from *ad-3A* mutants do not. It is concluded that the synthesis of phosphoribosyl-amino-imidazole succinocarboxamide synthetase is controlled by the *ad-3A* locus in *N. crassa.*

Reference

[1] C. R. Fisher, *Biochim. Biophys. Acta,* in press.

4.4 ENZYMOLOGY OF THE *ad-1* AND *ad-2* MUTANTS OF *SACCHAROMYCES* AND THE *ad-6* AND *ad-7* MUTANTS OF *SCHIZOSACCHAROMYCES*

C. R. Fisher

The genetic utility of the pigmented adenine-requiring mutants of *Saccharomyces* and *Schizosaccharomyces* has been limited, as in *Neurospora*, by a lack of information at the enzymic level. The techniques developed in *Neurospora* studies were applied to these two yeasts with complete success.[1] The enzyme phosphoribosyl-amino-imidazole-succinocarboxamide synthetase [5′-phosphoribosyl-4-carboxy-5-aminoimidazole: L-aspartate ligase (ADP) EC 6.3.2.6] from both yeast sources showed characteristics similar to the *Neurospora* enzyme. When synthetase activity was assayed in *ad-1* and *ad-2* strains of *Saccharomyces* and in *ad-6* and *ad-7* strains of *Schizosaccharomyces*, it was found that the *ad-1* and *ad-7* strains lacked synthetase activity, whereas the *ad-2* and *ad-6* strains possessed synthetase activity. This demonstrates that the synthetase is coded for by the *ad-1* locus of *Saccharomyces*

and the *ad-7* locus of *Schizosaccharomyces*. The methodology developed in our *Neurospora* studies provides a means of preparing these enzymes for studies at the molecular level.

It is interesting to note that the presence or absence of allelic complementation shows the same correlation with enzyme activity in all three organisms (Table 4.4.1).

Reference

[1]C. R. Fisher, *Biochem. Biophys. Res. Commun.*, in press.

Table 4.4.1. Summary of Locus-Enzyme Relationships and Intragenic Complementation

Organisms and Loci	Locus-Enzyme Relationship[a]		Allelic Complementation
	AIR ⇋ CAIR ⇋ SAICAR		
Neurospora crassa			
ad-3A	–	+	Negative
ad-3B	+	–	Positive
Saccharomyces cerevisiae			
ad-1	–	+	Negative
ad-2	+	–	Positive
Schizosaccharomyces pombe			
ad-6	+	–	Positive
ad-7	–	+	Negative

[a]The plus sign indicates that the enzyme is encoded by this locus; minus indicates that the enzyme is not encoded by this locus.

5. Drosophila Cytology and Genetics

E. H. Grell	
Biochemical Genetics (5.1–5.3)	**Chromosome Behavior (5.4–5.7)**
E. H. Grell Ruby D. Wilkerson Elizabeth S. Von Halle[a] T. G. Wilson[b,c]	Rhoda F. Grell C. W. Hinton[a]
[a]Consultant [b]Dual Assignments [c]Senior Trainee	

5.1 DETECTION OF CATALYTICALLY INACTIVE MUTANT ENZYMES ON THE BASIS OF HYBRIDS WITH ACTIVE ENZYMES IN *DROSOPHILA MELANOGASTER*

E. H. Grell

Chemically induced mutations of the genes specifying the structures of two enzymes, alcohol dehydrogenase and α-glycerophosphate dehydrogenase, have been produced. Homozygotes of these mutations have no detectable activity for their particular enzyme; however, the abnormal polypeptide may be detected for many mutant alleles. This detection is based on the formation of hybrid enzymes. A *Drosophila* heterozygous for two electrophoretic variants produces the two parental types of enzymes and hybrid molecules which have electrophoretic mobility between the parental types. In the hybrid enzyme one polypeptide subunit is specified by one allele and the other by the second allele.

Heterozygotes of ethyl methanesulfonate-induced mutations and an active allele contain the enzyme of the active allele and, with 70% of the mutations, also a hybrid enzyme. The hybrid contains an active polypeptide and a mutant polypeptide. Two mutant subunits would not be catalytically active, but one mutant and one active polypeptide can form active enzyme. In some cases a change in the net charge on the polypeptide accompanies the inactivating mutation. In the 30% which do not form hybrid molecules, there are at least three possible explanations. Possibly an abnormal polypeptide is not synthesized, it will not associate, or the hybrid does not have catalytic activity.

5.2 MUTANTS OF *DROSOPHILA* LACKING α-GLYCEROPHOSPHATE DEHYDROGENASE ARE INCAPABLE OF SUSTAINED FLIGHT

E. H. Grell

Methods for obtaining ethyl methanesulfonate-induced mutations of the gene specifying the structure of α-glycerophosphate dehydrogenase (GDH) have been described. Most of these mutants have no detectable GDH activity, although one mutant contains a small amount of activity (less than one-tenth of normal). Normal *Drosophila* are able to fly for many minutes and cover distances of many hundreds of centimeters. It is difficult to use distance that they travel as a measure of flying ability, since they tend to hover at one spot for fairly long periods of time. Mutants without GDH activity do not hover but can be stimulated to make short flights. After they fly a distance of about 50 cm they are not able to continue. Attempts to fly accomplish only low hops. The most reasonable biochemical explanation is that the enzyme is a member of the glycerophosphate cycle. This cycle regenerates NAD

from NADH. When the cycle is interrupted by the absence of a member enzyme, the passage of molecules through the glycolytic path is slowed by the shortage of NAD. After initial supplies of ATP are exhausted, the animal cannot fly because of the slow rate of glycolysis.

5.3 MALATE DEHYDROGENASE ISOZYMES OF *DROSOPHILA MELANOGASTER*

E. H. Grell

Drosophila has a collection of malate dehydrogenases (MDH) which is fairly typical of animals. There are two NAD-linked enzymes and a NADP-linked enzyme. One of the NAD-linked enzymes is present as a family of two and sometimes three isozymes. A genetic variant of this type has been found in a laboratory stock. The entire family of isozymes is more negatively charged than the usual type. Heterozygotes form hybrid enzymes, which indicates that there are two subunits in these enzymes. The genetics of the normal and variant MDH have been investigated. They are specified by a pair of alleles on the second chromosome at a genetic map position of about 41.2. An x-ray-induced deficiency of this MDH has been selected. The cytological limits of this deficiency are from 31A to 32A on Bridges' salivary chromosome map. The deficiency contains no *Mdh* allele and no *J* (jammed wing) allele. Flies heterozygous for the *Mdh* deficiency are small, develop slowly, and are female sterile. Homozygotes are lethal in an early stage. Whether the abnormal phenotype of the heterozygote is the result of the absence of the *Mdh* locus or some other absent gene is being investigated.

5.4 INDUCTION OF ANEUPLOIDY BY TEMPERATURE

Rhoda F. Grell

Nondisjunction of the meiotic chromosomes leading to aneuploidy and frequently to lethality is a well-recognized consequence of irradiation. It now appears that an increase in temperature of 10°C, delivered to female germ cells of *D. melanogaster,* is at least as effective in this respect as 4000 r of x rays. Unlike radiation, where brooding techniques have shown that sensitivity encompasses late oogonial stages as well as all stages of oocyte maturation up to diakinesis or metaphase I, the temperature response is confined to a discrete, rather brief period which may include the earliest oocyte. Thus, if the temperature for developing females is raised from 25 to 35°C during their sixth day (120–144 hr), the rate of primary X nondisjunction as calculated from their exceptional X progeny shows a twentyfold increase from a control value of 0.13 to 2.47%. Since nondisjoining chromosomes are most frequently noncrossover chromosomes, an increase of this magnitude might indicate an increased pool of noncrossover X tetrads (E_0's). If so, a Y chromosome, present in treated females, should expose the existence of this pool by pairing distributively with the X E_0's and diverting a large portion of them into secondary X exceptions. In accordance with this prediction, females carrying the dominantly marked B^sY were found to show an increase in secondary nondisjunction from 5.0% in the control of 23.3% in those treated from 114 to 138 hr.

To estimate the size of the pool of X E_0's present in a population of treated females, tetrad analysis of crossover data from such females has been carried out. The results (Table 5.4.1, lines 5 and 6) reveal that the frequency of E_0's for females carrying two normal X chromosomes and treated from 114 to 138 hr rises to 45% as compared with a control value of 6.8%.

For a more precise resolution of the heat-effective period, a 12-hr treatment, initiated at 114, 120, 126, 132, and 138 hr, was given to different groups of females. As shown in Table 5.4.1, lines 7 to 11, the frequency of primary X nondisjunction for the first three periods was 0.7% in each case; no primary exceptions were observed for the last two periods. In conformity with nondisjunction values, tetrad analysis of the X crossover data for each treated group showed increased frequencies of X E_0's in the first three periods and a return to normal and below-normal values for the last two periods respectively.

Although these studies establish that heat treatment, instituted at 114 up to 126 hr post egg laying, increases the number of noncrossover X tetrads, the increase in primary X nondisjunction requires clarification. Complex X inversions, present heterozygously, effect higher frequencies of X E_0's than those observed here, but X nondisjunction remains essentially unaltered. The simultaneous presence of other noncrossover chromosomes of proper size for recognition by the X's, such as the Y chromosome, is a prerequisite for increasing X nondisjunction. An explanation is provided if it is assumed that the heat-promoted increase in E_0's is not confined to the X chromosomes but affects the autosomes as well. Moreover, this assumption also explains the increased efficiency of the 24-hr treatment (120–144 hr) despite the lack of X response during the second half of the period. If the period of maximal E_0 induction for the X's precedes that for the autosomes, maximal interaction should occur when the heat treat-

Table 5.4.1. Effects of Heat Treatment on X Nondisjunction and Noncrossover X Tetrads

Genotype of ♀	Age at Treatment (hr)	X Nondisjunction (%)	E_0 X Tetrads (%)	Number
X/X		0.13		3061
X/X	120–144	2.5		890
X/X/B^SY		5.0		683
X/X/B^SY	114–138	23.3		215
y^2 *sc car*•y^+/y^2 *cv v f*		0.0	6.8	1621
y^2 *sc car*•y^+/y^2 *cv v f*	114–138	2.4	45.0	1019
y^2 *sc car*•y^+/y^2 *cv v f*	114–126	0.7	22.6	1139
y^2 *sc car*•y^+/y^2 *cv v f*	120–132	0.7	30.8	882
y^2 *sc car*•y^+/y^2 *cv v f*	126–138	0.7	20.9	839
y^2 *sc car*•y^+/y^2 *cv v f*	132–144	0.0	8.9	1541
y^2 *sc car*•y^+/y^2 *cv v f*	138–150	0.0	2.5	718

ment covers both periods. Tests for heat-induced autosomal nondisjunction are in progress.

The finding of heat-induced nondisjunction raises the possibility that variations in environmental temperature, particularly in the case of lower animals, as well as physiological temperature changes, perhaps incident to disease in warm-blooded animals including man, may contribute to the production of various kinds of aneuploidy.

5.5 THE DISTRIBUTIVE MODEL AND w^{m4}

Rhoda F. Grell

Merriam,[1] on the basis of an investigation of a particular homozygously inverted X chromosome, has challenged the validity of the distributive-pairing model of meiosis for *D. melanogaster*.[2] His conclusion rests upon the claim that a Y chromosome, introduced into females homozygous for the X inversion w^{m4}, both reduces X crossing-over and increases the frequency of noncrossover X tetrads; and further that this reduction can be eliminated to the extent that a nonhomologous competitor can be induced to pair with the Y and thus reduce X-Y pairing prior to exchange. The distributive model, by contrast, predicts that associations between the nonhomolog and the Y would not affect X exchange since they would be initiated subsequent to exchange.

A reexamination of w^{m4} (Fig. 5.5.1), including the distal third of the chromosome, heretofore largely neglected, has failed to confirm any of Merriam's findings. Results show that total crossing-over in w^{m4} homozygotes is unaffected by the presence of the Y (XX, 52.7 ± 0.6; XXY, 52.2 ± 0.7). Regional analysis shows that the Y causes a significant decrease in the proximal third between *wy* and *cv* (XX, 20.8 ± 0.5; XXY, 17.5 ± 0.5), no significant change medially between *f* and *wy* (XX, 14.2 ± 0.4; XXY, 12.9 ± 0.5), and a significant increase in the distal third (XX, 17.7 ± 0.4; XXY, 21.8 ± 0.6). The distal increase compensates

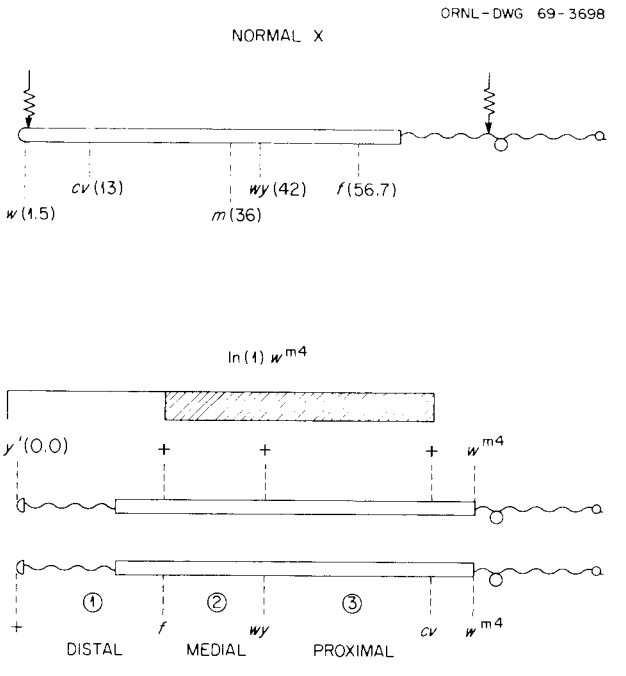

Fig. 5.5.1. Three Regions of Crossing-Over Measured in Present Study. Barred block over chromosome map indicates area studied by Merriam.

for the proximal decrease. Tetrad analysis, based on data from 8000 progeny from XX mothers and 5600 progeny from XXY mothers, gives a frequency of 12.4% E_0's for the former and 12.1% E_0's for the latter.

If the proximal decrease arises from competitive X-X-Y pairings, then the introduction of a nonhomolog which acts as a successful competitor for the Y should restore proximal X values, providing Y-nonhomolog associations precede exchange (Merriam model); proximal values should remain reduced if such associations follow exchange (distributive model). Three different competitors have been tested. A free fourth chromosome, which segregates from the Y 95.4%, shows a proximal X reduction by the Y of 4.3% when the competitor is present and 4.5% when it is absent; a free second chromosome duplication, which segregates from the Y 94.5%, gives reductions of 1.2% when present and 0.7% when absent; a multiply inverted second chromosome, SM1, which segregates from the Y ~ 92%, gives reductions of 3.8% when present and 3.2% when absent.

Segregation analysis, which permits an independent assessment of the two models, discloses that if the Y were specifying the segregational pattern of potential E_0's before exchange, pairwise associations of the Y greatly exceeding the maximal 100% would be required for each competitor tested. Thus, results from both exchange and segregation are incompatible with any model which specifies that segregational decisions be made preceding exchange but are entirely consistent with the distributive model.

References

[1] J. R. Merriam, *Genetics* **57**, 409–25 (1967).

[2] R. F. Grell, *Proc. Natl. Acad. Sci. U.S.* **48**, 165–72 (1962).

5.6 PHENOTYPIC REVERSION OF MUTANT TO WILD TYPE BY TEMPERATURE

Rhoda F. Grell

The gene for crossveinless (cv^+) is located at 13.7 crossover units from the tip of the X chromosome of *D. melanogaster*. The single mutant allele known (cv) removes both the anterior and posterior crossveins of the wing and shows complete penetrance. When males carrying the mutant cv are subjected to an elevation of 10°C in temperature (from 25 ± 0.5°C to 35 ± 0.5°C) for 24 hr during a specific period in development, a reversion to the wild cv^+ phenotype is observed. The frequency of reversion depends upon the age of the male at the time of treatment. When treatment is initiated during the third instar larval period at 107 hr post egg laying, the frequency of reversion is approximately 100%; treatment initiated at 114 hr produces ~90% frequency of reversion; whereas treatment initiated during the early pupal period at 136 hr leads to only 4% frequency of reversion.

Progeny tests of the reverted males show that the change is not a heritable one. It is possible that the sensitive phase represents a period of activity of the cv^+ gene product and that the heat-induced change in activity is accomplished through a temperature-dependent alteration in the conformation of the gene product.

5.7 CROSSING-OVER AND SEGREGATION IN TRANSLOCATION HETEROZYGOTES

Rhoda F. Grell

A break in each of two nonhomologous chromosomes accompanied by an exchange of the broken pieces at the time of rejoining produces a reciprocal translocation. When such an interchange is present heterozygously with the two normal homologs, a variable amount of aneuploidy results. Theoretically, two conditions are necessary for assuring balanced gametes. First, a multivalent comprising all of the involved chromosomes must be maintained until the first meiotic anaphase. Second, segregation from the multivalent must be of the alternate type, which means that the translocated chromosomes must go to the one pole and the normal ones to the other. A variety of evidence indicates that segregation from a translocation heterozygote is not random but, rather, favors the recovery of euploid gametes. The mechanism responsible for such preferential segregation has not been entirely elucidated, but crossing-over seems to be a prime requirement both for the retention of the multivalent and for alternate segregation.

To more fully understand the factors responsible, a series of reciprocal translocations have been under study in *Drosophila*. In each case the translocation has occurred between the right arm of chromosome 3 and the base of chromosome 4 so as to produce a large element called T_3 and a smaller acrocentric called T_4. The size of T_4 depends on the position of the break in 3R. The translocations were so chosen as to constitute a series in which T_4 becomes increasingly smaller (Fig. 5.7.1). First, a careful study of crossing-over throughout T_4 provided the data for a tetrad analysis. In this way the frequency that T_4 was a noncrossover tetrad

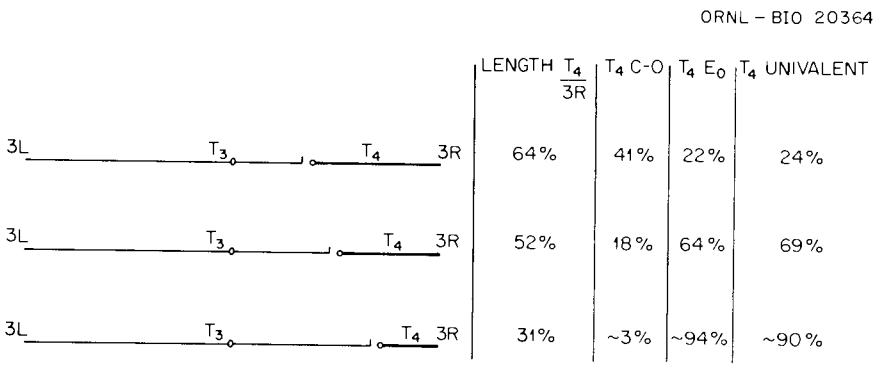

Fig. 5.7.1. Correlation Between the Frequencies that T_4 Is a Noncrossover Tetrad (E_0) and the Frequency that It Undergoes Pairing with a Nonhomologous Y Chromosome.

(E_0) could be calculated. Second, through an independent method it was possible to estimate the frequency that T_4 was a univalent and not part of the translocation configuration. This method makes use of the knowledge that chromosomes involved in distributive pairing with a nonhomolog are invariably noncrossovers. By introducing a marked Y chromosome into females heterozygous for the translocation, the frequency of nonhomologous association between the Y and each T_4 was estimated from segregation data.

The results show that the frequency that T_4 is a univalent and is free to pair and segregate from a nonhomolog corresponds very closely with the frequency that T_4 is a noncrossover (Fig. 5.7.1, columns 3 and 4). Furthermore the frequency that T_4 is a noncrossover is correlated with its length (Fig. 5.7.1, columns 1 and 3). As T_4 becomes smaller, the frequency that it is an E_0 increases, and the frequency that it pairs and segregates from the nonhomolog shows a parallel increase.

The fact that every T_4 that has failed to undergo exchange is a univalent which becomes involved with a nonhomolog has implications concerning the role of synapsis and exchange in segregation. Either noncrossover T_4's have never synapsed or synapsis was not followed by exchange. The first alternative requires the unlikely assumption that when synapsis occurs it is inevitably followed by exchange, regardless of the length of the chromosome involved. Furthermore, this alternative is refuted by studies of X duplications possessing much shorter sequences of homology than that present in the T_4's. Such duplications pair successfully as evidenced by the decreases they impose in exchange (up to 67%) between two normal X's, specifically in their region of shared homology.[1] If the second alternative is correct and T_4 E_0's represent that segment of T_4's which have paired but failed to undergo exchange, we must conclude that synapsis does not suffice to preserve associations until metaphase as postulated by Novitski.[2] Rather, synapsed chromosomes, in the absence of exchange, do fall apart and may subsequently participate in a second pairing. The reason that the asymmetry of the translocation heterozygote provides an initial clue to the amount of aneuploidy that may follow is that it suggests the amount of exchange that may be expected in each arm.

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[1] R. F. Grell, *J. Cell Physiol.* **70**, suppl. 1, 119–45 (1967).

[2] E. Novitski, *Genetics* **50**, 1449–51 (1964).

6. Yeast, Hymenoptera, and Phage Genetics

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Yeast Genetics (6.1)

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6.1 MUTATOR ACTIVITY OF X-RADIATION-SENSITIVE STRAINS OF *SACCHAROMYCES*

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Introduction. – We have reported that a radiation-sensitive strain of the yeast *Saccharomyces cerevisiae* has a higher spontaneous mutation rate than the control strains. The enhancement is greatest in mutations presumed to be of the addition-deletion type.

Several questions were unresolved at the time of our last report:[1] (1) we had noted low viability of some of the strains and wondered if this influenced our mutation rate determination by enhancing mutant selection, (2) we wanted to know if auxotrophic mutations which were presumed to be addition-deletion mutations behaved like the suppressors in the mutator system, and (3) we wanted to examine the spontaneous mutation rates of different radiation-sensitive strains. We have just begun our investigations on other radiation-sensitive strains.

Results. – Stocks of the wild type and radiation-sensitive strain, *xs*, were grown at 30 and 23°C. Cell viability measurements were made either by counting and plating or by separating the cells on agar slabs with a micromanipulator. The results are shown in Table 6.1.1. If stocks are grown at room temperature the cell viability of both the control and radiation-sensitive strains is noticeably higher. Consequently, for most of our more recent experiments the strains were grown at room temperature before inoculating into compartmented boxes for determination of spontaneous mutation rates.

The spontaneous mutation rates of the control and radiation-sensitive strain are shown in Table 6.1.2. The reversion test was on lysine-limiting medium. Each reversion was analyzed to determine whether it was a reversion of the nonsense mutation or the induction of

Table 6.1.1. Viability of Yeast Cells as Shown by Cell Separation and by Counting and Plating

Strain	Temperature Stocks Were Grown At (°C)	Temperature Inoculated At (°C)	Frequency of Viable Cells	Plating Efficiency
Control	30	30	0.684	0.805
xs	30	30	0.700	0.551
Control	23	30	0.988	0.983
xs	23	30	0.738	0.840
Control	23	23	0.950	0.937
xs	23	23	0.750	0.939

Table 6.1.2. Spontaneous Mutation Rates for Reversion from Lysine Requirement

Strain	Temperature of Incubation (°C)	Mutation Rate	
		Reversions at Locus	Induced Supersuppressor
		$\times 10^{-8}$	$\times 10^{-8}$
Control	30	0.44	4.04
	30	0.71	3.82
xs	30	1.09	19.35
	30	1.01	18.98
Control	23	0.59	5.12
xs	23	1.23	29.48

Table 6.1.3. Spontaneous Mutation Rates for Reversion from Uracil Requirement

Strain	Number of Compartments	Cells per Compartment	Number of Compartments Containing Mutants	Reversions per Nucleus per Generation
Control	1251	13.2×10^6	0	0
xs	1195	5.53×10^6	163	1.29×10^{-8}

a supersuppressor. It is evident from the table that most of the mutation rate enhancement is restricted to the supersuppressor class. Also it can be seen that the temperature of incubation had little effect, if any, on the spontaneous mutation rates.

As a final part of the study of the *xs* mutant strain, we obtained two auxotrophic mutants from G. E. Magni which are believed to be addition-deletion mutations. These are $ur_{4\text{-}11}$ and $thr_{3\text{-}10}$. New stocks were synthesized incorporating these two mutants. The mutation rates for $ur_{4\text{-}11}$ are shown in Table 6.1.3. It is obvious that when $ur_{4\text{-}11}$ is together with *xs*, its reversion rate is increased markedly. The spontaneous mutation rates for $thr_{3\text{-}10}$ have not yet been accurately measured, but, like $ur_{4\text{-}11}$, the spontaneous reversion index is clearly higher for the $thr_{3\text{-}10}$ when it is together with *xs*.

Thus, by every criterion so far studied, the enhanced mutation rate in the mutator strains seems to be associated primarily with addition-deletion mutations.

Our research is being extended to spontaneous mutation rates of other radiation-sensitive strains. It is worth noting that a strain sensitive to both x radiation and ultraviolet radiation, *uxs*, is also a mutator. Again, presumed addition-deletion mutations are the types of mutation that are enhanced in the radiation-sensitive strain. In one study with this strain the nonsense mutation reverted at a rate of 0.22×10^{-8} and the supersuppressor appeared at a rate of 17.1×10^{-8}. This is similar to the rate observed in the *xs* mutant strain (Table 6.1.2).

Discussion. – These mutator strains are the first that have been described as mutators for addition-deletion mutations. Mutators found previously have been described as specific for transitions[2] or transversions.[3]

Experiments under way indicate that the addition-deletion mutator, *xs*, is recessive in action. This implies the mutator responds as the loss of a gene activity, not as a change in its specificity. We assume that premutations occur frequently in the genome of the wild-type strain but that most of these are repaired.

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6.2 GENOME-DEPENDENT EXPRESSION OF TEMPERATURE-SENSITIVE MUTATIONS IN *HABROBRACON JUGLANDIS*

Roger H. Smith

Conditional lethal mutations are mutations that are lethal under one set of circumstances but not under another. These mutations are a result of single amino acid changes in a gene product, reflecting a single base-pair substitution in a gene.[1] The most common type of conditional lethal mutation that can be studied easily in higher organisms is the temperature-sensitive mutation. Suzuki *et al.*[2] have pointed out the possible use of these mutations in a variety of genetic analyses. We decided to study those expressing lethality at 35°C and complete viability at 30°C.

We were astonished to find that the majority of the temperature-sensitive lethal mutations become either wild type in expression or non-temperature-sensitive lethal mutations after one generation.

Habrobracon males of the Raleigh strain were fed 0.01 and 0.005 *M* ethyl methanesulfonate (EMS) in 0.5 *M* sucrose. They were kept at 30°C for 24 hr and then mated to females of the Lumberton strain with the genetic markers cantaloup eye color (*c*) and honey body color (*ho*). Daughters from this cross isolated as virgins were set for oviposition on host *Ephestia* larvae at 30°C. Virgin females produce only haploid male progeny. Thereby the entire genome can be screened for mutations in the F_2 generation. Eggs laid overnight (~16 hr) were allowed to develop at 30°C. The females were held without host larvae for 4 hr, then allowed to oviposit for 4 hr. These eggs were placed at 35°C for development. Egg counts, hatchability, and adult survival were scored for all F_2 progeny from each F_1 female. Females heterozygous for temperature-sensitive lethal mutations expressed at any time during development were easily detected. When a temperature-sensitive lethal was detected by this method, ten F_2 males reared at 30°C were backcrossed to virgin females of the Lumberton strain. Each virgin female resulting from this cross was then tested for the presence of a temperature-sensitive mutation. It was at this point that the instability in expression of these genes was first noticed.

Table 6.2.1 presents the results of testing the inheritance of 44 different temperature-sensitive lethal mutations. It is apparent that the expression of a large proportion of these mutations changed when attempts were made to transmit them through the second generation. Although some of these mutations could have been lost by chance sampling of only males which carried the wild-type allele, the recovery of unconditional lethals as well as the loss of expression of so many temperature-sensitive mutations provides a strong argument that a change in the genetic background drastically alters their expression.

Table 6.2.1. Results of a Test of Transmission of 44 Different Temperature-Sensitive Recessive Lethal Mutations

Recoverable as Temperature-Sensitive Lethals	Recoverable as Unconditional Lethal Mutations	Nonrecoverable Mutants
8	8	28

The F_1 females, heterozygous for the temperature-sensitive lethals, and hybrids between two different strains of *Habrobracon,* were probably heterozygous for many loci. When the F_2 males were backcrossed to females of the Lumberton strain, the change in the genetic background was apparently enough to alter the expression of these genes.

We suggest this simple explanation for our results. Since temperature-sensitive lethal mutations still produce entire protein subunits, it appears that shifts in the genetic background suffice to either totally activate or inactivate the gene product.

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6.3 TETRAPLOID FEMALES IN *MORMONIELLA*[1]

R. M. Macy[2] P. W. Whiting

In the hymenopteron *Mormoniella,* polyploidy has been recorded as occurring by mutation three times, once as a diploid male and twice as a triploid female.[3] A stock, Po-850, descended from one of the latter has been used extensively in genetical and cytological studies. Complementary allelism, involving five genes in the present test[4] (the eye color, oyster white, *oy,* scarlet, *st,* and peach, a double recessive, *pe•st,* called briefly, *pe,* an eye shape, glass, *gl,* and a body color, purple, *pu*), serves to separate polyploids from their sibs and tetraploid females from their triploid sisters.

There are 5 atelomitic chromosomes in the normal haploid males, 10 in the normal diploid females[5] and in

Fig. 6.3.1. Tetraploid Oocyte in Premetaphase. 2250X.

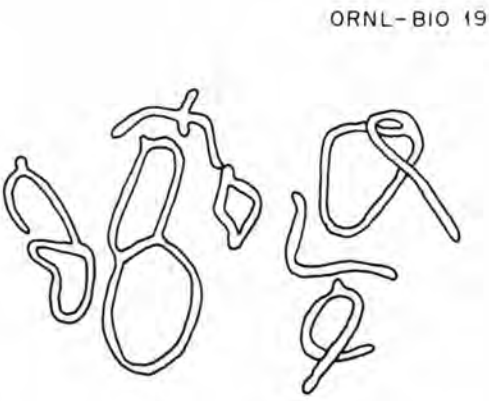

Fig. 6.3.2. Camera Lucida Drawing of Tetraploid Oocyte in Premetaphase.

the diploid males,[6] 15 in triploid females (unpublished), and 20 in the tetraploid females of the present study.

In spermatogenesis, there is no true synapsis and only one nuclear division, so that sperm of diploid males contain 10 chromosomes.[6]

In the maturation of eggs there are two events the condition of which may cause preferential segregation with respect to parental origin. The first is meiotic division of the oocyte, and the second is orientation on the meiotic spindle. If genes associated in the parents tend to segregate together, there is preferential segregation; if they are distributed at random, there is not.

Whiting[3] and Conner[7] demonstrated preferential segregation due to orientation on the spindle, and Conner proved preferential segregation in the oocyte division of triploid females.

The present study indicates no preferential segregation due to spindle orientation but highly significant preferential segregation due to meiotic division favoring parental combination in oogenesis of tetraploid females.

Chromosomes in oogenesis of tetraploid females were studied cytologically.[4] Figure 6.3.1 is a photograph of an oogonial tetraploid in premetaphase, and Figure 6.3.2 is a camera lucida drawing of the same cell. They show that the material is very suitable for detailed chromosome analysis. (In the drawing, chromosomes have been displaced to avoid overlapping.) For example, the chromosomes, equivalent to 20 univalents, appear from left to right as a quadrivalent (a ring of two homologs, with two homologs projecting laterally), a quadrivalent (a "figure 8" configuration), four bivalents, and a quadrivalent (another ring of two homologs with two homologs projecting laterally).

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7. Cellular Radiobiology and International Activities

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7.1 INTERNATIONAL ACTIVITIES AND CONFERENCES

Alexander Hollaender

Cooperation with Latin America. – The proceedings of the seventh Latin American symposium which was held in Caracas December 4–8, 1967, have appeared as a special issue of *Photochemistry and Photobiology.* They were edited by Dr. James Longworth of this Division and are now being distributed. It is a very impressive volume and has been very successful.

The eighth annual Latin American research conference was held December 1–6, 1968, at Belo Horizonte in the State of Minas Gerais, Brazil, on "Nuclear Physiology and Differentiation." Four members of the Division took part in this: Alexander Hollaender, Oscar L. Miller, Jr., Elliot Volkin, and R. C. von Borstel. The proceedings of this symposium will be edited at the University of Texas and come out as a special issue of *Genetics.*

Plans for the 1969 conference on "Fertility of the Sea" have been made; it will be held in Sao Paulo, Brazil, December 1–6. The 1970 conference on "Processing in the Vertebrate Visual System" will be held in Santiago de Chile. The 1969 conference will be cosponsored by Duke University and the 1970 one by Johns Hopkins University. These symposia have been uniformly successful and have had a profound influence on the development of basic biology in Latin America.

Conferences. – A round-table conference was organized in cooperation with the National Cancer Institute on carcinogenesis and molecular biology. It was held at Woods Hole, Massachusetts, June 24–28, 1968. It was a very successful conference, and the summary proceedings have been reproduced and distributed to members of the conference. No outside publication is planned.

7.2 CHEMICAL MUTAGENESIS IN *ASPERGILLUS*

Alexander Hollaender Vera M. Piper

A program on chemical mutagenesis was started using mutations in *Aspergillus terreus* as an indicator of the intensity of mutagenic action of chemicals. A list of the chemicals tested and a typical graph of the killing curve as well as mutagenesis curve are given in Table 7.2.1 and Fig. 7.2.1 respectively. It turns out that *Aspergillus terreus* is considerably less sensitive than *Neurospora* in regard to mutagenic response, and the concentrations of chemicals needed to get equivalent to *Neurospora* response is about five to ten times the concentration. This work is being continued and probably will go into an organism that is more sensitive than *Aspergillus terreus.* However, terreus will continue to be used as a standard check on other organisms.

Table 7.2.1. Survival and Mutation of *A. terreus* Conidia After Treatment with Various Mutagens

MNG = *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
ENNG = *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine
⬡–NH_2 = cyclohexylamine
Captan = 4-cyclohexene-1,2-dicarboxylic acid, imide, *N*(tetrachloromethylthio)
EMS = ethyl methanesulfonate
MMS = methyl methanesulfonate

Mutagens					
Compound	Concentration	Time	Percent Survival	Percent Mutations	Number of Experiments
Uv irradiation		20 min	~0.002	60	11
H_2O_2	1%	20 min	0.1	0	2
MNG	0.2 m*M*	22 hr	10	60	7
ENNG	0.8 m*M*	22 hr	10	15	3
⬡–NH_2	0.05%, high pH	5 hr	1	0	2
Captan	0.53 m*M*	24 hr	50	0 (?)	2
EMS	1%	22 hr	~0.1	40	5
MMS	0.17%	6 hr	0.2		4

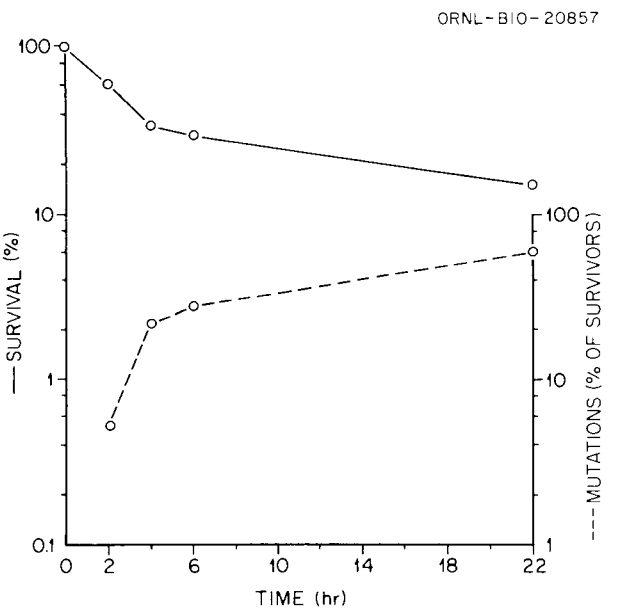

Fig. 7.2.1. Survival and Mutation of *Aspergillus terreus* Spores Treated with 0.2 m*M* MNG.

8. Radiation Microbiology and Microbial Genetics

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8.1 PROPERTIES OF DNA TRANSFERRED TO MINICELLS DURING CONJUGATION WITH VARIOUS DONOR *ESCHERICHIA COLI* STRAINS

Amikam Cohen Roy Curtiss III
W. D. Fisher[1] H. I. Adler

We have reported that the DNA-deficient minicells formed by *Escherichia coli* P678-54 can act as recipients of DNA during conjugation with F^+ donor cells.[2] We have now extended these observations to include transfer from donors of short and long chromosome segments (F' and Hfr types). Minicells can readily be separated from donor cells by centrifugal techniques, thus permitting the isolation of the transferred DNA immediately following mating. DNA isolated from minicells following mating with F^+ donors was separated by neutral CsCl gradient centrifugation into two fractions. Single-stranded DNA accounts for 50 to 80% of the total DNA, and double-stranded DNA accounts for the remainder. Incubation of minicells following mating resulted in apparent conversion of most of the single-stranded DNA to double-stranded DNA. DNA isolated from minicells following mating with Hfr donors or F' donors that transfer long chromosome segments consists of more than 95% single-stranded material. Only a small fraction of this latter DNA is converted to double-stranded structures during postmating incubation of minicells.

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8.2 ATTEMPTS TO FIND DNA-DEPENDENT RNA POLYMERASE IN MINICELLS

H. I. Adler Alice A. Hardigree

It has been reported that the DNA-deficient minicells formed by divisions near the poles of *E. coli* P678-54 also lack the DNA-dependent RNA polymerase.[1] We have been able to confirm this for minicells produced and harvested under a variety of conditions. From these observations and other evidence, we can suggest that most molecules of the DNA-dependent RNA polymerase normally remain in close association with DNA.

Few or none of them are free to diffuse out into the cytoplasm near the poles of the rod-shaped *E. coli* cells, and they are therefore not included in minicells. We considered the possibility that ultraviolet light (2650 A) might alter DNA sufficiently to interfere with the binding of the polymerase, but these experiments have been uniformly negative. We also prepared a minicell-producing rifampicin-resistant strain. In this strain it is likely that the RNA polymerase is structurally altered.[2] We postulated that this structural alteration might interfere with the normal binding of the polymerase to DNA, but we have not been able to find the enzyme in the cytoplasm of minicells produced by the rifampicin-resistant strain. The sum of our experience is that under no conditions do minicells contain this enzyme.

It is important for us to establish this fact in order to explain the failure of intact minicells to carry out protein synthesis. This inability to carry out protein synthesis has been observed both for minicells lacking DNA and for those containing small amounts of DNA introduced as a result of conjugation.[3] We have established and reported elsewhere that extracts of minicells are capable of producing protein-like materials when provided with a suitable message.[4] Therefore it seems reasonable to attribute the failure of protein synthesis in intact minicells to the absence of a message-generating system.

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8.3 GROWTH AND DIVISION OF INDIVIDUAL CELLS OF *ESCHERICHIA COLI* OBSERVED WITH TIME-LAPSE CINEMATOGRAPHIC TECHNIQUES

H. I. Adler Alice A. Hardigree

Using techniques previously described,[1] we have produced approximately 15 filmed sequences in which it is possible to follow growth, nuclear separation, and cell division in individual cells of an F^- bacterium growing on nutrient agar at 37°C. Cell growth is estimated by measuring the length of the photographically projected image as a function of time (40 frames correspond to 2 min). The width of the cells does not change significantly. We are currently interpreting the data obtained by this procedure as reflecting linear cell growth over approximately three-fourths of the generation time. During the last fourth of a generation time, there is an acceleration in the rate of cell elongation. This acceleration may not reflect a true increase in rate of synthesis. It probably reflects a stretching of the cell necessary to accommodate its contents, since the central constriction of cell wall and membrane (signifying the beginning of cell fission) is also initiated at this point in the generation cycle.

Most newborn cells contain two nuclear masses, and, in favorable sequences, the separation of these masses can be followed and related to growth and fission. Each nuclear mass separates into two bodies. The separation of the nuclei may be asynchronous, but at least one nucleus has initiated its division before the central constriction of cell wall and membrane begins. The distribution of daughter nuclei to daughter cells does not follow a strict pattern. A newborn daughter cell may receive nuclear material from both parental nuclei or may receive all of its nuclear material from one of the two parental nuclei. These two patterns of distribution occur with approximately equal frequency. Preliminary observations on other F^- K-12 strains and two *E. coli* B/r cultures suggest similar patterns of events.

Reference

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8.4 EARLY STAGES DURING CONJUGATION IN *ESCHERICHIA COLI* K-12

Roy Curtiss III L. G. Caro[1]
D. R. Stallions D. P. Allison[1]

The transfer of chromosomal material from donor to recipient strains of *Escherichia coli* K-12 must be preceded by at least three events: (1) interaction of donor and recipient cells to form unions that are stable during dilution (specific pair formation), (2) establishment of a conjugal bridge (effective pair formation), and (3) preparation of the donor chromosome for linear transfer (chromosome mobilization). Our studies were initiated to learn more about these events and to optimize conditions for their occurrence during bacterial conjugation. We found that donor cultures grown anaerobically, as opposed to aerobically, prior to mating have (1) a higher mean number of F pili per cell, (2) longer F pili, (3) a higher probability of forming specific pairs with F^- cells, and (4) a faster rate of

initiation of chromosome transfer. The growth medium for the donor culture also influences these same parameters: a rich medium is superior to a completely synthetic medium. Starvation of donor cells in buffered saline or for a required amino acid results in (1) a loss of F pili, (2) a loss in the ability of donor-specific phages to adsorb, (3) a loss of ability to form specific pairs with F^- cells and to yield recombinants, and (4) an increase in recipient ability. These changes occur as a function of starvation time, and the rates at which they occur are dependent on the conditions of prior growth and starvation of the donor culture. These observations provide a rapid method for the production of F^- phenocopies from donor cultures. Resynthesis of F pili commences very soon after restoration of normal growth conditions to a starved donor culture, but full restoration of donor ability, as measured by recombinant yield, occurs at a slower rate. We propose optimal conditions, based on our results, for achieving high mating efficiency, and we find, along with other investigators, that F pili are essential for specific pair formation. We also find, however, that the presence of F pili is not sufficient for display of donor ability, nor is the absence of F pili enough for cells to exhibit recipient ability. We suggest therefore that one or more components, in addition to F pili, are necessary for the conversion of specific pairs to effective pairs (and/or for chromosome mobilization) and also for preventing donor cells from acting as recipients.

Reference

[1] Biophysics section.

8.5 REQUIREMENT FOR EFFECTIVE HOMOLOGOUS PAIRING TO OBTAIN GENETIC TRANSFER DURING CONJUGATION IN *ESCHERICHIA COLI* K-12

Roy Curtiss III

Early appearance and maximum frequencies of inheritance of donor markers into recombinants in Hfr X F^- matings require homology between the region of donor chromosome first transferred and the comparable segment of the F^- recipient chromosome. To determine whether homology between the lead region of the donor chromosome and the comparable segment of the recipient chromosome was necessary for chromosome transfer and/or integration of transferred donor material, studies on episome transfer to recipient strains were initiated as a direct measure of genetic transfer. The F′ *lac*$^+$ and F′ *lac*$^+$ *proB*$^+$ *proA*$^+$ episomes have DNA contents equivalent to 2.8 and 5.5% of the chromosome respectively. F′ *lac*$^+$ transfer is unaffected by deletions or inversions in the *proB-lac* segment of the recipient chromosome, whereas F′ *lac*$^+$ *proB*$^+$ *proA*$^+$ transfer is reduced 100-fold by deletions of the *proA-proB* segment but is unaffected by deletions of the *proB-lac* segment of the recipient chromosome. Recombinant production resulting from chromosome mobilization by F′ *lac*$^+$ and F′ *lac*$^+$ *proB*$^+$ *proA*$^+$ is significantly reduced when the F^- possesses either the *proB-lac*$^-$ or the *proA-B*$^-$ deletion. F′ *thr*$^+$ *leu*$^+$ transfer is unaffected by deletions in the *proA-lac* segment of the recipient chromosome. It is inferred from these results that (1) all donor strains, be they F^+, F′, or Hfr, are capable of transferring several percent of their genetic material into F^- recipients without a requirement for effective homologous pairing, and (2) further transfer of Hfr chromosomes and long F′ episomes requires that the lead region of the donor genome transferred become effectively paired with the comparable region of the recipient chromosome.

8.6 PROBABILITY OF F INTEGRATION AND FREQUENCY OF STABLE Hfr DONORS IN F^+ POPULATIONS OF *ESCHERICHIA COLI* K-12

Roy Curtiss III D. R. Stallions

Integration of the fertility factor F into the chromosome of *Escherichia coli* K-12 results in the formation of an Hfr donor. We have determined the probability of stable F integration per bacterium per generation and per chromosome per generation and the frequency of stable Hfr donors in populations of the prototrophic type I F^+ strain χ209 by replica plating over 10^5 F^+ colonies to selective media spread with an F^- culture. (Stable F integrations were operationally defined as those which give rise to isolable clones of stable Hfr donors.) This method allowed detection and isolation of Hfr donors transferring the selected marker on the distal 20% of their chromosomes and present at frequencies from 1 to 100% in F^+ colonies. Thirty-four Hfr strains were obtained, representing ten unique marker transfer gradients. The frequency of pure clones of stable Hfr donors was 1.7×10^{-4}, and the probabilities of stable F integration were 3×10^{-6} per bacterium per generation and 8×10^{-7} per chromosome per generation. Matings between a multiple-marked F^- strain and an equal mixture of 12 Hfr strains having unique marker transfer gradients were conducted to determine the mean recombination frequency per Hfr cell for any chromosomal marker. Matings between this F^- strain and χ209 were also

performed to determine the mean recombination frequency per cell. By using these mean recombination frequencies and the frequency of stable Hfr cells in the χ209 population, we calculated that 15% of the recombinants formed in $F^+ \times F^-$ matings are due to stable Hfr donors in the F^+ population. Another 1% of the recombinants formed in $F^+ \times F^-$ matings are accounted for by stable integrations of F which occur just before or during mating. Therefore 80 to 90% of the recombinants formed in $F^+ \times F^-$ matings must be due either to unstable and/or lethal integrations of F into the chromosome or to a mechanism of chromosome transfer which does not require F integration into the donor chromosome.

8.7 GENETICS OF BACTERIA IN VIVO AS RELATED TO MICROBIAL ECOLOGY

R. T. Jones Roy Curtiss III

For studies on the importance of genetic exchange among bacterial species in nature, a large number of Enterobacteriaceae species have been collected from numerous strains of mice and examined for the presence of fertility factors and other episomes which mediate conjugal transfer of genetic material. By using specifically contaminated germfree mice (gnotobiotic), we plan to study the occurrence of genetic exchange in vivo with a system which approaches the ecological complexity of that found under natural conditions. As a prelude to the above studies, *Escherichia coli* K-12 was chosen to obtain some basic information in in vivo genetic exchange. *E. coli* K-12 substrain χ820 was selected as the F^- recipient for these studies because it possesses mutations that are well distributed about the circular genome of *E. coli* and that show low reversion frequencies both in vivo and in vitro. Contamination of germfree mice with χ820 followed by addition of Hfr, F′ or F^+ K-12 strains results in the appearance of all recombinant classes at frequencies that would be expected in an in vitro mating experiment. Inheritance of unselected donor markers occurred at frequencies that were dependent on linkage relationships established in in vitro experiments. With time, certain recombinant classes predominate, while others decline. In vitro and in vivo growth experiments using different combinations of recombinant types indicate that environmental selection may play an important role in determining which recombinant classes persist.

One of the *E. coli* strains of mouse origin (strain DXM2) used in initial studies was found to gain ascendancy over other *E. coli* strains following mixed infection of germfree mice. Although remaining genetically stable during most in vitro experiments, this organism became genetically unstable in vivo. High mutation frequencies in sugar fermentation and phage resistance were noted. We have preliminary evidence that the genetic instability of strain DXM2 observed in vivo is not manifest by contamination of germfree mice with DXM2 alone but requires the presence of other bacterial species or strains. At present we do not know whether this effect is strain and/or species specific. By using conjugation with various *E. coli* K-12 Hfr strains having different origins for initiation of chromosome transfer, we have shown that DXM2 *mal*$^-$ mutations (inability to ferment or utilize maltose) arising in the mouse intestine occur in both the *malA* and *malB* loci. Loci adjacent to the *malA* and *malB* loci, based on the genetic map of *E. coli* K-12, do not appear to be affected. Differences in back-mutation frequencies from *mal*$^-$ to *mal*$^+$ further indicate that many of these *mal*$^-$ mutations are of independent origin. Also, we are studying the in vitro growth rates and generation times of the parental DXM2 strain and of *mal*$^-$ mutants isolated from the mouse. We now have evidence that strain DXM2 carries a resistance transfer factor which confers resistance to >150 μg/ml of terramycin. This element appears to be of the *fi*$^+$ type in that it inhibits the function of the fertility factor "F" of *E. coli* K-12. The RTF can be eliminated from the host strain by treatment with Acridine Orange. We are currently studying RTF transfer to other *E. coli* strains in vitro and in vivo and are determining whether this factor can cause chromosome transfer.

8.8 POSSIBLE STREPTOMYCIN RESISTANCE FACTOR IN *PROTEUS MIRABILIS* AND ITS EFFECT ON RECIPIENT ABILITY DURING CONJUGATION

G. J. Stine Annie S. Angel
Roy Curtiss III

Wild-type strains of *Proteus mirabilis* lack the ability to ferment lactose (*lac*$^-$). However, it is possible to transfer a *lac*$^+$ gene into *Proteus* by conjugal mating with strains of *Escherichia coli* which have their *lac*$^+$ gene attached to the fertility factor F. This is referred to as an F prime *lac*$^+$ (F′ *lac*$^+$).

In order to develop our own system for the transfer of a variety of different F primes from *E. coli* into *Proteus mirabilis*, an *E. coli* F′ *lac*$^+$ *str*^{S} (streptomycin sensitive) strain was mated with a *lac*$^-$ *str*^{r} mutant strain of *Proteus mirabilis* (selected to be resistant to 200 μg of streptomycin per milliliter). A large number of *str*^{r} *E. coli* colonies were found among the *Proteus*

lac$^+$ recombinant colonies appearing on the streptomycin selective media. Analysis for streptomycin sensitivity of the parent wild-type *Proteus* strain (POR-4) revealed that the POR-4 culture carried three cell types with respect to sensitivity to streptomycin. There were cells sensitive to 5 and cells resistant to 25 and 50 μg of streptomycin per milliliter. A similar study on a wild-type strain of *Proteus mirabilis* (PM-1) received from Dr. L. S. Baron revealed that PM-1 contained two cell types, those resistant to 25 and those resistant to 50 μg of streptomycin per milliliter. Growth of the PM-1 strain in the presence of Acridine Orange resulted in the derivation of PM-1 cells that were sensitive to 5 μg of streptomycin per milliliter. Conjugal matings were then performed to determine if there was a difference in the frequency of F′ gene transfer into the *Proteus* cell types that differed in their sensitivity to streptomycin. An auxotrophic *E. coli* F′ *lac*$^+$ strain was mated with each of the derived strains. Recombinant frequencies for the *lac*$^+$ gene of *ca.* 10^{-5} resulted from matings using the streptomycin-sensitive POR-4 and PM-1 and their streptomycin-resistant (200 and 400 μg/ml) lines. *Lac*$^+$ recombinant frequencies obtained in matings with the low-level streptomycin-resistant strains and their high-level streptomycin-resistant mutants (200 and 400 μg/ml) were *ca.* 10^{-7}. It is thus possible that the low frequency of recombinants obtained in matings between *E. coli* F′ donors and mutants of the original *P. mirabilis* strains could have been due to the presence of a resistance transfer factor in the original *P. mirabilis* strains that conferred low-level streptomycin resistance.

8.9 ISOLATION OF *ESCHERICHIA COLI* EPISOME DNA FROM *PROTEUS MIRABILIS*

G. J. Stine Annie S. Angel
Roy Curtiss III

Many auxotrophic mutants have been isolated from *Proteus mirabilis*, and some of these have been used as genetic recipients in mating experiments with *Escherichia coli* donor strains which carry specific F′ factors. As a result of these mating experiments, we now have strains of *P. mirabilis* carrying the following F′ factors or episomes from *E. coli:* F′ *lac*$^+$ (200PS and ORF-207); F′ *lac*$^+$*proC*$^+$*purE*$^+$ (ORF-1); F′ *his*$^+$ (F′ 3513); F′ *argG*$^+$*str*^{s}*malA*$^+$ (KLF-41); F′ *trp*$^+$ (Col *trp*); F′ *thr*$^+$*ara*$^+$*leu*$^+$ (F′-038); F′ *gal*$^+$ (F′18); and F′ *lysA*$^+$*thy*$^+$ (KLF-16). *P. mirabilis* DNA has a G-C content of 38%, whereas *E. coli* DNA has a G-C content of 50%. Therefore, following extraction of DNA from these hybrid strains, the *E. coli* F′ DNA can be separated from the *P. mirabilis* DNA by cesium chloride density gradient centrifugation. It is thus theoretically possible to fractionate the entire *E. coli* chromosome. At this time, two DNA fragments of the *E. coli* chromosome (F′ *lac*$^+$ and F′ *lac*$^+$*proC*$^+$*purE*$^+$) have been isolated from the *P. mirabilis–E. coli* F′ hybrids. We are continuing to introduce F′ factors of various lengths and containing different segments of the *E. coli* chromosome into *P. mirabilis* to obtain a complete set of overlapping DNA fragments for the entire *E coli* chromosome.

9. Mammalian Cytogenetics

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9.1 RESTRICTED REUNION OF X-RAY-INDUCED LESIONS AT THE LEVEL OF THE CHROMOSOMAL SUBUNIT

J. G. Brewen W. J. Peacock[1]

Early autoradiographic studies on the types and frequencies of sister chromatid exchange (SCE) indicated that the functional chromosomal subunit involved in these exchanges had directional polarity. The molecular component of the chromosome that satisfied this restriction of directional polarity was the single polynucleotide chain of DNA. Hence it was concluded that the functional chromosomal subunit was single-stranded DNA. Subsequent studies on SCE frequencies have raised doubt as to whether their formation is restricted by the property of directional polarity of the chromosomal subunits. These results thus make suspect the conclusion that the functional subunit is single-stranded DNA.

Since we had shown earlier that SCE was primarily a radiation-induced event, we designed the following experiment to test, independent of analysis of SCE, the hypothesis that the functional chromosomal subunit has directional polarity by analyzing the subunit rejoining patterns of x-ray-induced chromosomal aberrations.

Figure 9.1.1 summarizes the experimental theory. Following incorporation of the isotope, each chromatid will contain one labeled and one unlabeled segregating subunit. Proximal union following isochromatid breakage induced at this time will result, after suppression of anaphase with Colcemid, in a "mirror-image" dicentric chromosome in the succeeding cell division. The pattern of label segregation in the dicentric will depend on whether there is a restricted rejoining of the breaks. Restricted rejoining may be of two types: (1) labeled-to-labeled and unlabeled-to-unlabeled association of the subunits will result in a dicentric having all the label conserved in one sister chromatid in the portion between the two centromeres, barring sister chromatid exchange (Fig. 9.1.1*A*); (2) labeled-to-unlabeled association of the subunits will result in dicentrics all having a switch of label at the midpoint between the two centromeres (Fig. 9.1.1*B*). Random association of the subunits will yield equal frequencies of these two label patterns.

One hundred thirty-seven "mirror-image" dicentrics from polyploid cells were analyzed. In 104 of them, all of the label was conserved in one sister chromatid in the intercentromeric region. In 27 of the remaining 33 chromosomes, the label switches were in positions obviously distinct from the midpoint and were therefore considered examples of labeled-to-labeled subunit rejoining associated with a sister chromatid exchange that occurred either before or after the formation of the dicentric.

The remaining 6 chromosomes appear to have a label switch at their midpoint. These chromosomes may have

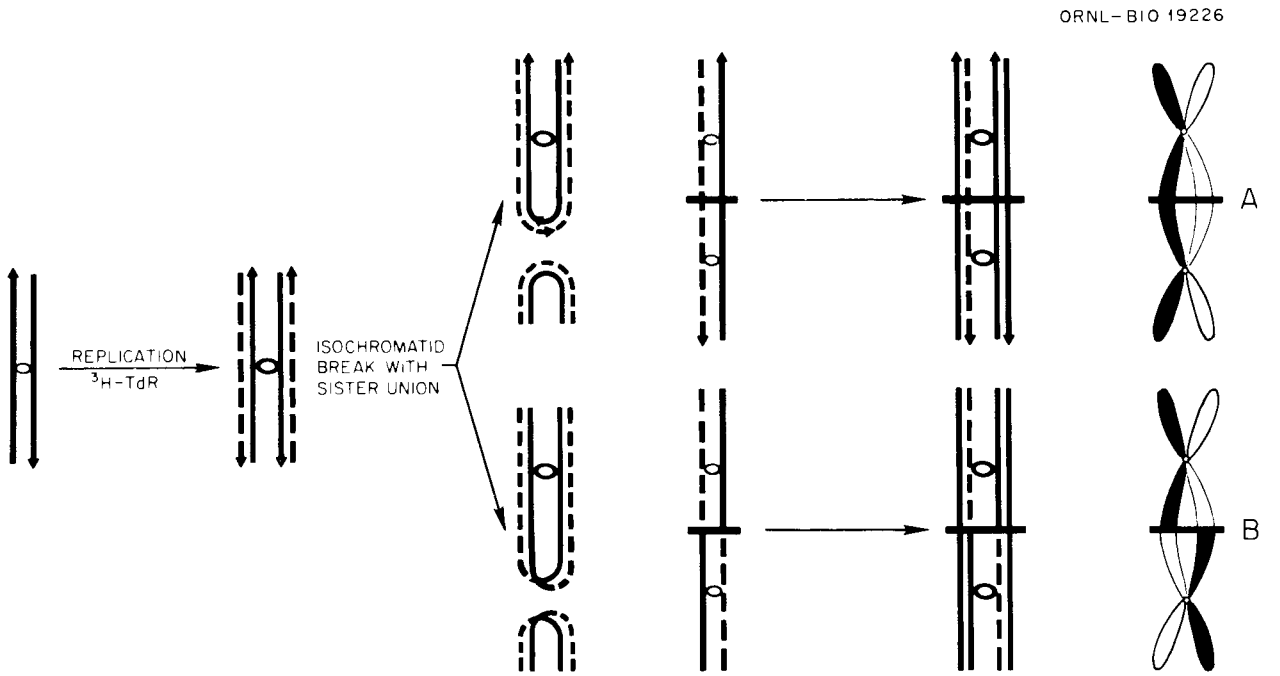

Fig. 9.1.1. Schematic Representation of Subunit Associations During Sister Union.

resulted from labeled-to-unlabeled union of subunits, but they can be accounted for on the restricted rejoining model, since with the observed frequency of 0.02 to 0.03 exchange per micron of chromosome length, 3 to 8 of a sample of 137 chromosomes can be expected to have midpoint exchanges. In this calculation the midpoint is defined by the limits of autoradiographic resolution, viz., between 0.5 and 1 μ on either side of the true midpoints.

These results agree with the hypothesis that there is a restriction in rejoining of chromosomal subunits such that the labeled subunit of one chromatid is able to rejoin only with the labeled subunit of its sister chromatid. This is precisely what is predicted if the subunits involved in these aberrations are single polynucleotide chains of DNA. We conclude therefore that the functional chromosomal subunit is one, or more, single strand of DNA and furthermore that the DNA is the primary target in aberration formation.

Reference

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9.2 MEIOTIC STUDIES IN THE CHINESE HAMSTER

J. G. Brewen F. G. Pearson
H. E. Luippold

A vast literature has accumulated on the effects of ionizing radiations and chemical mutagens on mammalian somatic chromosomes. An important rationale for this effort has been an attempt to assess mammalian, particularly human, radiation hazards in a genetic sense. Although data on somatic cells is very informative, the final analyses must be made on germ cells if direct knowledge is to be gained.

We have undertaken to study the effects of ionizing radiation and chemical mutagens on the germ cells of the Chinese hamster as measured in spermatogonial and meiotic chromosomes. These studies include the analyses of induced chromosomal aberrations as well as effects on crossing-over, as measured by chiasmata frequencies, with particular emphasis placed on when, in the cell cycle, the effects are most dramatic.

In order to conduct these experiments, accurate characterization of the normal cell cycle had to be

made and suitable techniques developed. To this end we have utilized previously published techniques and developed new ones to obtain suitable preparations of spermatogonial and meiotic chromosomes.

Particular emphasis has been placed on determining the normal chiasmata frequency, at diakinesis, in the Chinese hamster and the timing of the meiotic cycle by tritiated thymidine tagging of the chromosomes. Control animals have a chiasmata frequency of 15.55 ± 0.45 per cell and 5.10 ± 0.30 for bivalents Nos. 1 and 2. A series of animals were then injected, intraperitoneally, with tritiated thymidine (specific activity 6.0 curies/ml) at a concentration of 3 μc per gram of body weight, and one or two animals were sacrificed daily from the day of injection until 20 days later. Air-dry preparations were made of meiotic prophase figures and diakinesis analyzed for chiasmata frequencies. The results are summarized in Fig. 9.2.1. It is apparent that the ^{3}H-TdR had no effect on chiasmata frequency, although an occasional (<1%) chromosomal interchange was seen.

Preliminary results indicate that 100 r of x rays induce a significant (>5%) number of interchanges and affect the chiasmata frequency; however, no definitive statement on stage sensitivity can be made at this time, owing to the fact that the autoradiograms are still being exposed. It has also been observed that an injection of ^{3}H-TdR directly into the testes results in light, but uniform, labeling of the meiotic prophase chromosomes within 3 hr of the injection. These results are being pursued further.

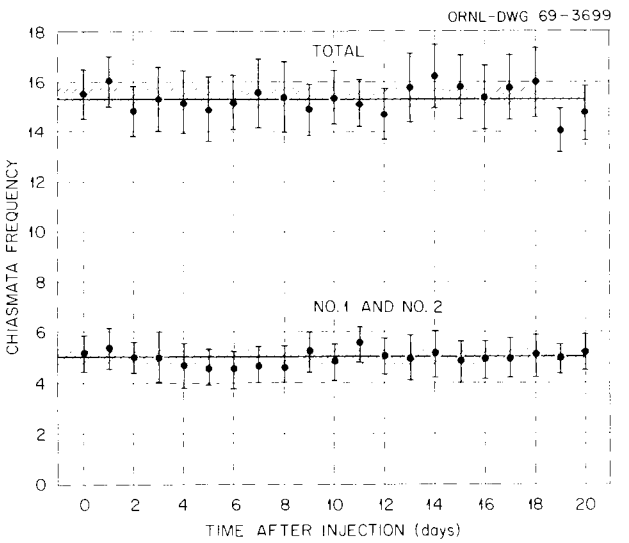

Fig. 9.2.1. Chiasmata Frequencies for the Entire Genome (Upper Line) and Bivalents 1 and 2 (Lower Line) at the Various Days After Injection of Tritiated Thymidine. Standard errors for the individual days are indicated. The straight lines represent the mean chiasmata frequencies for all samples.

It is hoped that further work will elucidate the sensitivity of the meiotic chromosomes to ionizing radiation in terms of chromosome breakage and chiasmata formation and that these results can be related to the cell cycle, with particular interest paid to the period when recombination occurs.

9.3 KINETICS OF TWO-BREAK ABERRATION PRODUCTION BY X RAYS IN HUMAN LEUKOCYTES

M. A Bender M. A. Barcinski[1]

H. J. Evans has repeatedly asserted that the production of two-break chromosome aberrations by x irradiation of human peripheral leukocytes in vitro is an approximately linear function of dose.[2] This is in striking contrast both to what has long been observed in other materials and to our own results[3] as well as those of other laboratories with irradiated human leukocytes. Evans suggested that the dose-square kinetics reported by others for ring and dicentric chromosome production are an artifact arising from fixation of the cells after longer times in culture than he allows. This explanation cannot be correct, however, because Norman and Sasaki[4] have reported dose-square kinetics in experiments in which the cells were fixed after even shorter times in culture than those used by Evans. As an alternative explanation, Evans suggested that the difference between his results and those of other laboratories might result from his use of his own modification of the standard leukocyte culture technique.[5] We have therefore repeated Evans' experiments, following his technique as closely as possible.

Whole-blood samples were collected with heparin from two normal, healthy volunteers. The cultures were irradiated 6 hr after they were set up. Two cultures from each donor were given exposures of 50, 100, 150, 200, or 250 r of 250-kv x rays at a rate of 50 r/min. One culture from each donor for each dose, plus an unirradiated control, was fixed after 52 hr in culture; a duplicate series was fixed at 56 hr.

A chi-square test showed no significant differences between the results for either fixation time or either donor, so we pooled the data for further analysis (Table 9.3.1). Least-squares regression analyses were done to determine the best fits of the deletion and of the ring and dicentric data to the linear ($Y = a + bD$, where Y is the yield, a the control frequency, D the dose, and b the coefficient of aberration production) and the

Table 9.3.1. Chromosome Aberration Yields from Pooled Data for Two Donors, Each with Fixations at 52 and 56 hr

X-Ray Exposure (r)	Cells Scored	Deletions	Rings	Dicentrics	Percentage Deletions[a]	Percentage Rings and Dicentrics[a]
0 (control)	397	5	0	0	1.3 ± 0.6	0
50	284	30	1	6	10.6 ± 1.9	2.5 ± 0.9
100	234	56	7	24	23.9 ± 3.2	13.2 ± 2.4
150	238	85	13	54	35.7 ± 3.9	28.2 ± 3.4
200	309	133	20	144	43.0 ± 3.7	53.1 ± 4.1
250	255	175	19	181	68.6 ± 5.2	78.4 ± 5.5

[a]Standard errors assuming a Poisson distribution.

dose-square ($Y = a + bD^2$) models. The dose-square model was rejected for "lack of fit" (chi-square test, $P < 0.01$) for the deletion data, as was the linear model for rings and dicentrics. The best-fit coefficients of aberration production were: $b = 2.48 \pm 0.12 \times 10^{-3}$ deletions per cell per r, and $b = 12.74 \pm 0.15 \times 10^{-6}$ rings and dicentrics per cell per r^2. The data were also fitted to the model $Y = bD^n$. For deletions, the best-fit value was $n = 1.01 \pm 0.08$; for rings and dicentrics, $n = 2.15 \pm 0.07$.

It is clear that we failed to confirm Evans' results, in spite of a careful attempt to duplicate his experimental conditions. Although we are at a loss to account for the virtually linear dose-effect kinetics observed by Evans, it is evident that neither the short culture time nor the particular culture technique he used is responsible.

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[1]Pan-American Health Organization Postdoctoral Fellow on leave from the Instituto de Biofiscia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brasil.

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10. Mammalian Genetics

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10.1 LACK OF EFFECT OF CAFFEINE ON REPAIR OF RADIATION-INDUCED MUTATION IN THE MOUSE

W. L. Russell R. B. Cumming
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The possible effect of caffeine on the induction of specific-locus mutations in male and female mice was tested in 1962 by Lyon, Phillips, and Searle.[1] Most of the data in the male involved germ cells that had been treated in dividing, spermatogonial, stages. The results provided no evidence for any induction of mutations by the caffeine treatment. No specific-locus mutations were obtained in 31,686 progeny from treated females, and the mutation rate in treated males (1 mutation in 32,463 offspring) is, in fact, lower than the spontaneous rate, although not significantly so.

The possibility, suggested by results from microorganisms and mammalian tissue cultures, that caffeine might interfere with the repair of radiation-induced

specific-locus mutations in the mouse had not been tested. In view of the extensive consumption of caffeine by man, it was considered important to explore this possibility.

(101 × C3H)F_1 female mice were treated with caffeine and exposed to 400 r of gamma radiation from a ^{137}Cs source. On the following day, they were mated to T-stock males homozygous for seven recessive visible mutations. The offspring were scored for new mutations at the seven marked loci. The radiation dose rate chosen (0.6 r/min) was one which permits a significant amount of repair of mutational damage, but not as much repair as occurs at lower dose rates. The rationale was that a repair mechanism operating at intermediate efficiency would be sensitive to modifying factors.

Caffeine was administered in two intraperitoneal injections. The first, given immediately before the beginning of radiation exposure, consisted of 200 mg per kilogram of body weight in approximately 1 cc of Hanks' Balanced Salt Solution. The second injection was half that amount and was delivered at the midpoint of the approximately 11 hr of radiation exposure time. Irradiation was interrupted for the few minutes required for this injection. Since the half-life of caffeine in the mouse is probably only a few hours, the double injection was designed to maintain a high concentration of caffeine in the body during the period when damage is occurring and repair could start.

Preliminary toxicity tests of caffeine had been made to determine the highest concentration practicable. With the dose used, a few animals died shortly after the second injection. A small pilot experiment, with the second injection three-quarters, instead of half, the amount of the first injection, resulted in too many deaths.

The total experiment has yielded only 2 specific locus mutations in 8019 offspring. If caffeine had interfered with repair of mutational damage to the degree that high radiation dose rate (without caffeine) is presumed to interfere with it, we would have expected 12.4 mutations (based on 23 mutations obtained in 14,842 offspring of females exposed to 400 r at 90 r/min). The observation of only 2 mutations is significantly below that frequency (P = 0.002). It is clear that caffeine is not interfering with the repair process to the extent that higher radiation dose rate does.

Is caffeine having any effect at all? Simultaneous controls without caffeine were not run. However, the results can be compared with data from earlier experiments made at the same dose of 400 r and the similar dose rate of 0.8 r/min. In those experiments, 30 mutations were observed in 71,056 progeny. If the current experiment had had the same mutation rate, 3.4 mutations would have been expected. The observed number of 2 is, of course, not significantly different from this.

It may be concluded that there is no evidence that caffeine in these experiments has had any effect at all on the repair process.

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10.2 SEARCH FOR INVERSIONS IN THE MOUSE; A PROGRESS REPORT

Liane B. Russell Kathren F. Stelzner
N. L. A. Cacheiro

Among the variety of chromosomal aberrations reported and studied in the mouse are translocations, insertions, deficiencies, duplications, and sex-chromosome losses and additions. However, inversions have not, so far, been recovered. Since such inversions would be valuable tools in cytogenetic studies, experimental mutagenesis, and population experiments, a search for them is being undertaken.

The general method is to screen for crossover suppression in offspring of irradiated mice and to investigate further by cytological means. Five marked linkage groups have been built up, but to date only three have been used.

Linkage-Group 20 (X Chromosome). – The search for X inversions presents problems not encountered in the case of autosomal inversions. The biggest of these is that cytological analysis must be made in female meiosis. In spite of this, considerable emphasis has been placed on this linkage group, since inversions in it would be invaluable tools in the study of X-chromosome inactivation.

To date we have analyzed about 200 sperm (F_1 sibships) from irradiated males yielding about 5000 F_2 progeny. Since only the male F_2 is used to determine crossover frequency and since one of the nonrecombinant classes turns out to be quite inviable, the spread of F_1 sibships with respect to recombination is great. Cytological analysis has been performed on the progeny of those sibships that gave less than 15% recombination between *Gs* and *spf*. Even though a good method for studying meiotic anaphase in females was developed by us, the work is made difficult by the fact that anaphase is of very short duration, and, even with hormone synchrony and careful control of variables, only about 10% of the eggs are in this

stage. No clear inversions have been found, although progeny from a sibship that gave 3.9% recombination (expected value about 30%) shows some cytological abnormalities in anaphase. Translocations have been found, but it is not yet known if they are X-linked.

Linkage Group 1. – About 2500 F_2 have been classified from 47 F_1 sibships. At least two autosomal translocations have been found. Four sterile F_1 males may carry Y-linked translocations. No inversions have yet been identified.

Linkage Group 5. – Only 20 F_1 sibships with about 600 F_2 have been analyzed to date, and no aberrations have been found so far.

Estimates based on frequency of nonrestituted breaks (calculated from translocations recovered in past experiments) indicate that inversions will be very rare. Thus the probability of inducing an inversion in any one chromosome with the dose used is probably about 0.06%. Inversions that did not include at least one of the markers would probably not be detectable by reduced recombination.

10.3 FUNCTIONAL HEMIZYGOSITY AND RECOVERY OF X-AUTOSOME TRANSLOCATIONS IN THE MOUSE

Liane B. Russell Clyde S. Montgomery

Our work with X-autosome translocations, T(X;A)'s, in the mouse led, in 1961, to the single-active X chromosome hypothesis and, two years later, to our modification of this hypothesis, according to which inactivating influences spread along gradients from an inactivation center or region in the X. In subsequent work genetic limits were placed on the location of this center or region.

It is to be noted that the postulated mechanism creates, in the T(X;A) female, an animal mosaic for functional hemizygosity of certain autosomal regions. Thus, where the inactivation center is in play in the intact X, all the autosomal alleles in the translocation (as well as in the normal autosome) are active; but where it is in play in the translocated X, autosomal alleles within a certain distance become suppressed, the ones in the intact autosome thus acting hemizygously.

Among the questions posed by this mosaic hemizygosity are: (1) Does it confer ill-effects? (2) What is its bearing on the frequency of recovery of T(X;A)'s? To contribute information on these questions, we have studied various vital statistics in about 8000 animals of five independent T(X;1) stocks, R2, R3, R4, R5, and R6. Comparisons involve translocation and nontranslocation segregants from crosses of T(X;1) females to intact males carrying appropriate markers. Translocation daughters are generally classifiable by variegation; and sons by color, sterility, or low testis weight.

The findings for a number of different measurements indicate that in heterozygous females, all T(X;1)'s have very definite, though perhaps not extreme, deleterious effects. That this is not due to loss of chromosomal material associated with the rearrangement is shown by the normality – in all respects (except for the chromosomally caused sterility) – of the males. In other words, mosaicism for functional hemizygosity of autosomal regions appears to be measurably deleterious.

This conclusion has a definite bearing on the frequency of recovery of X-autosome translocations. Although our translocations were by-products of specific-locus mutation rate experiments, rough calculations may be made to derive an estimate of the number expected on the simplified assumption that all chromosomes have an equal chance of being involved in a rearrangement. Such calculations are based only on acutely irradiated postspermatogonial stages (in which total induced translocation frequency has been established), and take count of the restrictions that one must have (1) X-bearing sperm, (2) one of only four marked autosomes involved, and (3) a break distal to the inactivation center in the X and proximal to the marker in the autosome, or vice versa. The number of X-autosome translocations found in about 40,000 tested germ cells is about one-fourth to one-half as great as that estimated on the above assumptions. Similarly, in making a specific attempt to produce and detect T(X;A)'s, Lyon and Meredith[1] failed to do so among 38 translocations and concluded that there was a shortage of such rearrangements.

Another interesting fact is the unequal distribution of the rearrangements among autosomes. Of nine now known in the mouse, six involve LG1 (linkage group 1), two involve LG8, and none involve the other two marked autosomes, namely LG2 and 5. One or more of the following factors could enter into this unequal distribution: (1) relative lengths of marked chromosomes; (2) relative positions of the inactivation center and autosomal marker (i.e., both distal, or both proximal); or (3) location, near some of the markers, of "critical" genes, that is, genes that must be active in double dose in all cells to allow survival. The information available about relative lengths of the four marked autosomes seems to indicate that the first of these is at least not the *only* factor. The second possibility becomes relatively less important if the inactivation center is fairly centrally located in the X, as in fact it appears to be.

As far as the existence of "critical" genes is concerned, positive evidence will be difficult to obtain, since their existence would normally result merely in absence of certain types. However, enough information exists to show that there is a whole range in tolerance to hemizygosity. At one extreme are perfectly viable deficiencies, of which many are known in the mouse. Next in order is total-body hemizygosity that is deleterious but not lethal; next, conditions that lead to lethality when total-body but that are compatible with life when mosaic; and next, the condition described here, that is, part-body functional hemizygosity that is deleterious. The final step, the existence of "critical" genes (i.e., genes for which part-body functional hemizygosity is lethal) thus seems plausible. It would account for the dearth of X-autosome translocations in general, as well as for the shortage of specific T(X;A)'s.

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10.4 EVIDENCE FROM X-AUTOSOME TRANSLOCATIONS CONCERNING THE NATURE OF X-CHROMOSOME INACTIVATION

Liane B. Russell Clyde S. Montgomery

Five X-1 rearrangements that arose at this laboratory provide an opportunity for studying the same loci in relation to different rearrangement points.

Genetic evidence indicating that the rearrangements studied are reciprocal translocations comes from breakpoint mapping, from the fact that no animals resembling type II *flecked* are found, from tests of presumed non-R recombinants, and from special tests in one of the rearrangements. Recovered recombination frequencies at medium distances from the breakpoints are not reduced and may be slightly enhanced; in the immediate vicinity of at least one of the breakpoints, recombination may be somewhat inhibited. Segregants with chromosomal imbalance, other than that involving an intact X, die prenatally. Translocation females only rarely, if ever, yield a segregation product containing the two translocated chromosomes plus the normal X. On the other hand, transmission of the intact LG1 (linkage group 1) alone (i.e., OX^P) is quite significantly higher than from normal females.

When single markers are used, *c* is inactivated more than *p* in some (R3, R4), but *p* more than *c* in others (R5, R6, R2); when both loci are marked together, the more strongly affected one may be inactivated alone, but the more weakly affected one is inactivated only in conjunction with the other (spreading effect). These findings are at variance with some suggestions (e.g., differential multiplication rates, different gene functions, or a diffusible gene product) that have been made by other authors to explain unequal amounts of *c* and *p* variegation. Instead, they confirm our earlier suggestion of a gradient of inactivation. The spread in both directions indicates that inactivation does not emanate from the X centromere; and mapping in conjunction with labeling experiments indicates that, in two of the rearrangements, R5 and R6, inactivated autosomal regions are associated with noncentromeric portions of X.

Attempts to determine whether the spread of inactivation occurs with equal ease through X chromosomal material as through autosomal material substituted for it will not be critical until genes with localized action are found in certain specific regions of the X. Some genetic and cytological evidence exists in favor of this idea but is far from exact at this time.

Decrease in inactivation with increasing distance from the inactivation center is the result of fewer cells being affected rather than of depression to an intermediate level. This completeness of inactivation, which produces mosaicism for functional hemizygosity, has already been used as a tool for studies of gene action and developmental genetics. It now presents a strong argument against Grüneberg's recent hypothesis[1] that partial inhibition of gene action occurs in *both* X chromosomes. Grüneberg's hypothesis has also been rejected on other grounds.

Reports of other T(X;A)'s with nonrandom differentiation made it necessary to examine the possibility of partial nonrandomness in the five T(X;1)'s studied. This can be ruled out in two of them, R3 and R6, where at least one autosomal substitution leads to 50% variegation. Preliminary cytological studies and other evidence make it somewhat unlikely for two others, R2 and R4. Ohno's[2] recent interpretation that random inactivation is possible only if "X-chromosome material as a unit is preserved" by the translocation can be rejected on the basis of breakpoint mapping in R6, a randomly inactivating T(X;1).

Evidence that it is neither the X centromere nor X-chromosomal material "as a unit" that determines inactivation further confirms our earlier hypothesis of an inactivation center or region, upon the location of which we have been able to put genetic limits. The T(X;1) results are at variance with Grüneberg's recent hypothesis of inhibition emanating simultaneously from both X's. They are completely consistent with the original idea that the inactivation center or region

comes into play in only one X chromosome, when two are present. Inactivation in this chromosome proceeds in both directions, the probability of a given locus being affected decreasing with distance, and the effect, when it does occur, being the equivalent of total inactivation.

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10.5 EFFECT OF RADIATION OF NEONATAL AND JUVENILE FEMALE GUINEA PIGS ON THEIR SUBSEQUENT FERTILITY

E. F. Oakberg Evelyn C. Lorenz

It is well established that in the female mouse, early dictyate oocytes are extremely sensitive to radiation and that even exposure to low doses and low dose rates results in marked effects on fertility. The possibility exists that other mammals also may have a period of high oocyte sensitivity during the period of transition from rapidly progressing meiotic stages of the fetus to the "arrested" stage characteristic of the adult.

The guinea pig was chosen for comparison with the mouse because both the cytological appearance and the radiation response of the "arrested" oocyte of the adult female differ markedly from that of the adult mouse. Also, the oocyte stages which occur during the transition from pachytene to the "arrested" stage during late fetal and neonatal life differ in the two species.

Female guinea pigs were given 300 r of 250-kv x rays at 0, 1, 7, 10, 14, and 28 days of age and were mated for lifetime fertility tests when five to eight weeks old.

There was no effect of radiation on number of litters born per female, litter size, nor proportion of young weaned. It can be concluded that the radiation response of the oocytes of juvenile guinea pigs differs significantly from that of immature mice.

10.6 THE SPERMATOGONIAL CELL CYCLE DURING REPOPULATION OF THE RADIATION-DEPLETED SEMINIFEROUS EPITHELIUM

E. F. Oakberg Evelyn C. Lorenz
Diane W. Slover

The ability of the radiation-depleted seminiferous epithelium to repopulate itself from less than 1% surviving type A spermatogonia involves a complex relationship between cell potentiality and control of spermatogonial differentiation. Three simple alternatives present themselves. (1) Formation of spermatogonia irreversibly committed to sperm production requires a certain local density of stem (type A) cells. Lacking this, differentiation is interrupted until the required population is built up by division of survivors. (2) Radiation merely strips off the sensitive, more numerous, late spermatogonial stages, leaving a basic stem cell population which then goes through a hitherto unrecognized pattern of multiplication and differentiation. (3) A combination of (1) and (2), involving survival of a small fraction of type A spermatogonia which constitute the true stem cell population and which first must multiply before normal stem cell renewal and differentiation are established.

We had observed previously that a higher percentage of spermatogonia were labeled by ^{3}H-thymidine five to seven days after high radiation doses than in controls. In order to evaluate possible explanations of this phenomenon, 101 X C3H hybrid male mice, 12 weeks old, were given either 500 or 1000 r in a single exposure, or two 500-r fractions 24 hr apart. Mice were injected with ^{3}H-thymidine five days after 500 r, and seven days after 1000 and 500 + 500 r exposures. Equal numbers of control and irradiated mice from each exposure were killed at intervals ranging from 30 min to 52 hr after ^{3}H-thymidine injection.

Our previous observation of higher labeling percentages in irradiated mice was confirmed. The values were 30% for control, 37% for 500 r, 41% for 1000 r, and 44% for 500 + 500 r groups. Controls showed the expected regular movement of labeled cells through mitosis, with two labeled mitotic peaks separated by an interval of unlabeled mitotic figures. In the 500- and 1000-r groups, labeled mitoses first appeared at the same time as in controls (i.e., the minimum value for G_2 was the same), but distinct peaks of labeled mitoses did not occur. Thus great variability in cell generation times is suggested. In the 500 + 500 r series, two discrete mitotic peaks of labeled cells, just as in controls, are suggested by the incomplete data.

The mitotic indices for 1000 and 500 + 500 r groups were lower than for 500 r and control mice. Thus, the cells labeled, but did not go through mitosis at the normal rate. Several hypotheses could be presented, but the data do not permit distinction between radiation effects on the cell cycle and the possibility that the survivors possessed inherently different generation times owing to their stage in stem cell renewal at the time of radiation.

11. Mammalian Recovery

C. C Congdon[a]

Secondary Disease (11.1–11.10)

C. C Congdon[a]
Eijo Endo[b]
A. L. Kretchmar[c]
W. H. McArthur[c]
Frances E. Hacker[a]
Alberta P. Henley
R. E. Toya, Sr.
W. R. Conover[d]
M. L. Davis[e]
M. R. Proffitt[f]
Joan L. Rasor

Recovery *in vitro* (11.11–11.14)

L. H. Smith
T. W. McKinley, Jr.
M. L. Clayton[g]

[a]Dual Assignments
[b]Visiting Investigator from Abroad
[c]Consultant
[d]Loanee
[e]USPHS Predoctoral Fellow on Leave of Absence from the Pathology and Physiology Section
[f]USPHS Predoctoral Fellow
[g]Student Trainee

11.1 THE RESPONSE SURFACE FOR MORTALITY FROM SECONDARY DISEASE[1]

C. C Congdon

Mortality from secondary disease in mouse radiation chimeras can best be visualized as a response surface created by known and unknown experimental factors. The known factors make up a factor space as shown in Fig. 11.1.1. The larger rectangular solid encloses the factor space for all experimental designs carried out or under way at the present time. The smaller inserted block approximates the factor space for design V and is a region that gave low 90-day secondary disease mortality in rat-mouse chimeras. Sex factors were also studied in all designs, and the most recent experiments, design VII, include two levels of environment as a fifth factor that might influence secondary disease mortality. A three-level genetic factor is also a fundamental part of the overall plan.

Future designs should probably include other obvious factors such as radiation exposure, age of host, and a more extended range for the factors of age of donor, cell dose, and genetic variation. The major interest at this time, however, centers on trying to identify unknown factors related to the environments surrounding the donor and host animals. A preliminary attempt to get at this is being made in design VII.

Results of the design studies can be summarized by describing the response surface for mortality as it is now understood. The low point or region of least mortality for both allogeneic and rat bone marrow transplantation is in that portion of the factor space shown by the insert. Delayed injection on day 1 or 2 after irradiation seems to be particularly important for low mortality. The surface rises sharply from the "well-like" low point so that greater mortality occurs in other areas.

In finding low and high regions of the response surface the goal is to pragmatically specify how to avoid mortality from the disease. The important additional need is to have experiments with high and low mortality so that metabolic and pathologic responses can be determined under these controlled conditions. This latter goal is essential to gain insight into the mechanisms involved in high and low mortality.

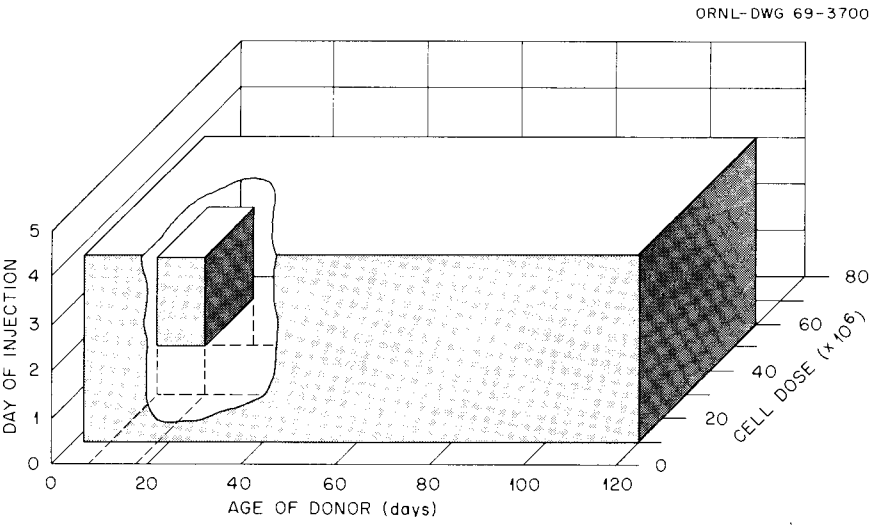

Fig. 11.1.1. Factor Space for Known Factors in Experimental Design. Region of factor space that gives low point in mortality response surface shown in insert.

Reference

[1] In collaboration with M. A. Kastenbaum of the ORNL Director's Division and T. J. Mitchell and D. A. Gardiner of the ORNL Mathematics Division.

11.2 LOCALIZATION OF LYMPH NODE LYMPHOCYTES IN ATROPHIC SPLEEN WHITE PULP OF IRRADIATED MICE

M. L. Davis

Introduction. – Previous studies have shown that small lymphocytes circulate through the body by selectively immigrating from the blood stream into lymphoid tissues (thymus excluded) and then recirculate to the blood via the lymphatics.[1-4] Although the route of circulating lymphocytes is well documented, properties of these cells which control their fate with respect to localization and recirculation are poorly understood. It is not known what proportion of the animal's total lymphocyte population recirculates and what proportion remains permanently or temporarily fixed in lymphoid tissues.

Results and Conclusions. – To study the localization of injected lymphocytes, lethally irradiated mice were given an intravenous injection of 50×10^6 lymph node cells obtained from either isogeneic, allogeneic, or xenogeneic donors. At short time intervals thereafter, recipients were sacrificed and nucleated cell counts made on recipient spleen cell suspensions. A maximum of 20% of the injected cell dose was found to localize in the recipient spleen by 12 hr after injection regardless of donor type. Mice receiving isogeneic cells maintained this spleen localization level through the following six days, whereas mice given allogeneic or xenogeneic lymphoid cells developed a graft-vs-host reaction visible histologically 24 hr after injection with a concurrent rise in spleen nucleated cell number.

When allogeneic lymphocytes were exposed to 10,000 r in vitro immediately prior to injection, these cells localized in the recipient spleen in numbers similar to unirradiated cells but on histologic examination were found primarily in the red pulp. Those cells that did localize in the (lymphoid) white pulp compartment did not undergo a graft-vs-host reaction. When allogeneic donors were presensitized with recipient-type spleen, greater numbers of cells were counted in the recipient spleen than with nonpresensitized donors cells. When irradiated recipients, presensitized to donor-type spleen 14 days earlier, were injected with donor lymph node cells, very poor localization was found.

These data indicate that normal circulation of lymphocytes through the body can be altered by changing the properties of these cells or the environment through which they pass.

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11.3 THE EFFECT OF POLYOMA VIRUS ON LYMPHATIC TISSUES

M. R. Proffitt

Introduction. – Recently it was shown that the Rauscher virus, which produces a murine leukemia, initiated a rather extensive and prolonged regenerative hyperplasia of lymphatic tissue germinal centers.[1] The hyperplasia lasted throughout the 40-day observation period, much longer that that seen with ordinary antigenic stimuli. Apparently this response is not peculiar to the Rauscher virus alone, for Notkins *et al.* made a similar observation with the LDH virus.[2] In the belief that this morphological feature may be related to the apparent immunosuppressive properties of various viruses,[3,4] including the Rauscher virus,[5] we are currently attempting to ascertain whether other proliferating antigens produce similar effects. Herein is contained a preliminary report of the effects of the oncogenic polyoma virus on lymphatic tissues.

Methods. – Twenty-five adult C3H female mice were injected ip with approximately 10^6 to 10^7 plaque-forming units of polyoma virus. The mice were autopsied at 1, 2, 4, 8, 16, and 32 days. To test for possible immunosuppression, one group of mice received 10^8 rat erythrocytes ip 15 days after injection of virus. Five days later, sera were taken from these animals and hemagglutinin titers were determined. In all instances, vehicle and nontreated control groups were included.

Results and Discussion. – In the polyoma group, spleen weights increased to 137 mg at day 8 compared with 88 and 86 mg for vehicle and untreated controls respectively. Spleen weights in the polyoma group subsequently declined and were only slightly above initial levels by day 32. Histologically, germinal center size and number per longitudinal spleen section rose sharply, reaching a maximum number of 59 in the polyoma group at day 16 compared with 21 and 13 in the vehicle and control groups respectively. Maximum germinal center size was reached on day 8 at which time the hyperplastic centers made up 50 to 70% of the white pulp. Lymph node germinal center changes were comparable with those in the spleen. The hemagglutinin titers to rat erythrocytes in the experimental as well as the control groups were found to differ little, all averaging about 1/128.

The assumption must be made that although polyoma virus does produce a markedly detectable change in lymphatic tissue germinal centers, it is a change probably not greatly different from that seen with normal antigenic stimulation and certainly not like that reported for the Rauscher and LDH viruses.[1,2] We further considered the possibility that the polyoma virus may be immunosuppressive. Indeed, one strain has been shown to enhance the oncogenicity of another strain under conditions of concomitant infection in vivo.[6] However, this suspicion appears not to have been substantiated, at least for the strain of virus tested and by the parameter measured.

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11.4 LIPID ACCUMULATION IN LIVERS OF MICE UNDERGOING THE GRAFT-vs-HOST REACTION

R. E. Toya, Sr.

Introduction. – The lethal effect of parental strain spleen cells injected into sublethally irradiated F_1 hybrid mice is well known. Fatty livers have been observed under these conditions,[1] but the accumulation of fat has been considered to be nonspecific in relation to the graft-vs-host reaction. Graft-vs-host disease is an immunologic reaction, and a pronounced fatty liver suggests there may be a relationship between liver lipid accumulation and severity of the graft-vs-host disease. It is indeed possible that liver malfunction is the basis for the early lethal effect of the graft-vs-host reaction and is the result of an immunological reaction. This work was undertaken in an attempt to quantitate the graft-vs-host reaction in terms of the amount of liver lipid.

Methods and Materials. – Male (C57BL/6 X C3H)F_1 mice were exposed to 800 r of x rays, and within 1 to 2 hr each mouse received an intravenous injection of 10×10^6 parental (male C3H) spleen cells. Treated animals were sacrificed at selected intervals, up to seven days after treatment, for total liver lipid determination.[2]

Results and Discussion. – In an initial mortality study it was noted that animals injected with 10×10^6 parent spleen cells died between days 7 and 9 following irradiation and cell injection and consistently showed a fatty liver; therefore total liver lipid was determined at this dose of spleen cells.

The gross appearance of the animals was similar to that seen in the foreign bone marrow reaction,[3] including weight loss, hunching of the back, and thinning and ruffing of fur. Except for the thymus, lymphoid organs were visibly enlarged, as were the splenic follicles, which gave the spleen a nodular appearance. A consistent finding with the fatty liver was an enlarged gall bladder. All animals were eating, although food intake was decreased as noted by intestinal and stomach contents at the time of sacrifice.

Values for total liver lipid are presented in Table 11.4.1. For the normal (C57BL/6 X C3H)F_1 mouse, liver lipid amounts to 4.8% of wet weight. For the irradiated F_1 mice injected with parent spleen cells, liver lipid increased to a maximum of 10.1% on day 6 and then declined slightly on day 7. X-ray control animals showed a slight increase over controls. After one and two days of starvation, liver lipid increased, an expected finding because it is known that starvation results in a fatty liver due primarily to increased mobilization of fatty acids from adipose tissue.

Fatty livers have been produced by chemicals, special diets, and anemia and can be brought about by any agent which causes (1) an increased synthesis of triglycerides, (2) decreased oxidation by the liver, (3) increased uptake of triglycerides or fatty acids from the blood, (4) decreased secretion of triglycerides by the liver, or (5) any combination of the above.

Davis *et al.*[4] used the sulfobromophthalein (BSP) test for liver function in sublethally irradiated mice undergoing the foreign spleen reaction and found that in animals with severe fatty livers there is a reduced BSP clearance, indicating impaired liver function and blockage of channels for lipid excretion.

Chemicals that produce a fatty liver do so by depression of hepatic protein synthesis, thus interfering with synthesis of lipoproteins which are required for the secretion of triglycerides by the liver, and a resulting accumulation of triglycerides occurs. In amino acid deficient diets there are indications that deficiency damages the oxidation processes in the liver, but this alone could not account for the fat accumulation. A significant relationship between the degree of anemia and fatty infiltration of livers in irradiated mice and rats has been shown.[5] It seems likely that these, and perhaps other factors, are involved in the graft-vs-host reaction resulting from the injection of parent spleen into irradiated F_1 mice.

Table 11.4.1. Total Liver Lipid in (C57BL/6 X C3H)F_1 Mice

Time After 800 r + 10×10^6 C3H Spleen Cells (days)	Number of Animals	Lipid[a] (%)
2	5	4.7 ± 0.1
3	5	5.2 ± 0.1
4	5	5.0 ± 0.1
5	5	6.4 ± 0.8
6	24	10.1 ± 1.0
7	4	7.1 ± 1.0
Time After Starvation (days)		
1	5	6.9 ± 1.2
2	4	10.7 ± 1.8
Normals	11	4.8 ± 0.2
800 r only, day 6	5	5.5 ± 0.4

[a]Values shown are percent of wet weight, ±1 standard error.

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11.5 EFFECT OF SYNGENEIC ANTILYMPHOCYTE SERUM ON RAT-MOUSE RADIATION CHIMERAS

Nazareth Gengozian[1]

Earlier studies showed that syngeneic spleen cells given to rat-mouse radiation chimeras caused a severe depletion of grafted hemopoietic elements and a subsequent seeding of syngeneic blood-forming cells. Because

the spleen cells provided both immunocompetent and hemopoietic cells, we investigated the possibility of creating a reversion to host-type blood-forming cells with antilymphocyte serum (ALS); sporadic spontaneous reversions are known to occur in this model. Mouse antirat lymphocyte serum was injected ip and subcutaneously to established chimeras at close intervals. All chimeras showed marked destruction of bone marrow and partial depletion of lymphatic tissues at autopsy. Peripheral blood examination during ALS treatment revealed marked depletion of lymphocytes and anemia. Deaths occurred at varying intervals ranging from day 1 after injection to day 28. Recovery of host-type bone marrow cells was not observed in two mice that lived a week or more following the last injection. Neither was rat cell recovery seen in the absence of the suppressive effect of ALS. Although reversion to mouse-type elements was not obtained to the present time, the results show that the syngeneic ALS used in this study had a marked destructive effect on grafted hemopoietic and lymphatic tissue.

Reference

[1] Medical Division, Oak Ridge Associated Universities; in collaboration with the Mammalian Recovery section.

11.6 INCREASED GROWTH OF *MYCOBACTERIUM LEPRAE* IN THE FOOT PADS OF THYMECTOMIZED-IRRADIATED MICE

C. C. Shepard[1]

We have confirmed the finding of Rees *et al.*[2,3] that when thymectomized-irradiated mice are infected in the foot pads, the local growth of *Mycobacterium leprae* is distinctly increased over controls. The mice, (101 X C3H)F_1 hybrids, were thymectomized at the age of three months, and nine days later were x irradiated with 950 r and injected intravenously with 5×10^6 syngeneic bone marrow cells. The treated mice and untreated controls were injected three days later with 5×10^3 *M. leprae*. In the foot pads of the controls, bacilli increased to a plateau level of about 10^6 at 131 days, where it remained for the duration of the experiment. The proportion of solidly staining (viable) bacilli fell to insignificant levels after 131 days. In the treated mice, bacillary growth during the early (logarithmic) phase was not any more rapid, but it continued beyond 131 days at a gradually slowing rate until it approached 10^8. The continuing growth was evidenced both by the increase in bacillary numbers and by the continued presence of solidly staining bacilli. In histopathological studies of the controls, *M. leprae* was not found outside of a local area of the inoculated foot, and nerve invasion was not seen; this was in agreement with previous experience in other lines of mice. In the thymectomized-irradiated mice the bacilli were observed to spread throughout the inoculated foot after 131 days; at 494 days and thereafter, bacilli were also found in the regional lymph nodes, in the other feet, and in the ears and nose. At 553 days and thereafter they were also found in the bone marrow of all the feet. Bacillary nerve invasion was frequent in the mice killed later, both in the inoculated foot and in the areas to which the infection had spread.

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11.7 PENETRATION OF INTESTINAL TISSUES BY *H. NANA* IN IMMUNIZED MICE

Wallace Friedberg[1] B. R. Neas[1] D. N. Faulkner[1]

In mice immunized to the intestinal parasite *H. nana*, it is not known whether the immune response to a challenge infection inhibits the parasites before or after they penetrate the intestinal tissue. To investigate this question we challenged immunized and nonimmunized mice with *H. nana* eggs and counted the parasites in the intestinal tissues 5 to 90 hr later. The challenge dose of 100,000 eggs was injected directly into the lumen of the duodenum. At 5 hr after the egg challenge, the median parasite count was 552 in the nonimmunized mice and 770 in the immunized animals. Ninety hours after the egg challenge the median parasite count (cysticercoid stage) was 3020 in the nonimmunized group and zero in the immunized mice. Thus in the immunized animals an appreciable number of parasites penetrated the intestinal tissue and then apparently were digested before they reached the cysticercoid stage. In tissue sections from the nonimmunized mice, 5 to 11 hr after the egg challenge, there was usually a space around the parasite and little or no cellular infiltration. In the immunized mice there was a cellular

infiltration, predominantly eosinophils and mononuclear cells, around the parasite.

Reference

[1] Civil Aeromedical Institute, Federal Aeronautics Administration, Oklahoma City, Okla.; in collaboration with the Mammalian Recovery section.

11.8 LYMPHOPOIESIS AND CELLULAR IMMUNITY IN THE MOUSE: COMPUTER AND EXPERIMENTAL STUDIES

Joan L. Rasor A. L. Kretchmar

Introduction. – To study effectively the varied aspects of lymphopoiesis and the role it plays in cellular immunity, a schematic model is proposed. This model consists of two subdivided units, the first of which is concerned with the production, recirculation, and destruction of lymphocytes in non-antigen-stimulated animals and the second with altered lymphopoiesis resulting from the presence of antigen. The conceptual model can be converted into mathematical statements and programmed into an analog computer. Studies with the computer performed so far have been with each unit separately.

Results and Discussion. – Experiments involving the rate of recovery following total-body x irradiation with leg shielding[1] were simulated on the computer. Results indicated that the interdependent populations of the non-antigen-dependent unit could start from a depleted condition, recover, and maintain a steady state. Two further studies with the computer, simulating thoracic duct cannulation[2] and extracorporeal irradiation,[3] were done to test the model. Computer studies closely approximated the published experimental data.

Studies on the antigen-dependent unit are still in the preliminary stages, but results suggest that the model will be able to account for the cellular events which occur following introduction of antigen into the system.

Experiments are currently under way to determine the rate of recirculation of lymphocytes in the mouse by injecting suspensions of lymphocytes into lymphocyte-depleted mice on the third day following whole-body x irradiation and taking peripheral blood samples for cell counts at various time intervals following the injection.

The proposed model for lymphopoiesis has been helpful in two ways. First, it has served as a means of effectively assessing the available, voluminous, and sometimes conflicting literature on the subject, and second, it has been suggested what areas need further experimental work. The model is not meant to be an end in itself but rather a means of studying in an orderly fashion the process of lymphopoiesis.

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11.9 EARLY PROLIFERATION OF TRANSPLANTED SPLEEN COLONY-FORMING CELLS

A. L. Kretchmar W. R. Conover[1]

Introduction. – Previous work[2] showed that the number of spleen colony-forming cells that can be obtained from spleens of irradiated mice given syngeneic bone marrow cells increases over the interval of 24 to 96 hr. The results suggest that proliferation among transplanted colony-forming cells begins early, probably during the first 48 hr after transplantation. The interpretation is rendered difficult, however, by a phenomenon observed earlier by others[3,4] and illustrated in Fig. 11.9.1.

Results and Conclusions. – 1×10^6 syngeneic bone marrow cells were given to all primary recipients. Spleen cells were then harvested from these animals at 1 or 4 hr and one, two, three, or four days and were injected into secondary recipients (one-half spleen equivalent per mouse). Secondary recipients were killed at nine days, and the number of colony-forming cells in spleens of primary recipients was calculated from the number of gross nodules found in spleens of secondary recipients. Data plotted in Fig. 11.9.1 show that there is a substantial decrease in the number of colony-forming cells in the spleen of primary recipients between 4 and 24 hr, followed by an apparent lag period and then a rapid increase between 72 and 96 hr.

Data summarized in Fig. 11.9.2 suggest that the decrease is due to circulation of colony-forming cells in the primary recipient. The effect of this circulation of cells appears to be a net emigration of cells from the lung and spleen to the bone marrow. The number of colony-forming cells in the spleen is, therefore, not a measure of the number of colony-forming cells in the animal until equilibrium of circulation has been achieved with the number of emigrants from the spleen balanced by immigration to the spleen. It is not known when such equilibrium is achieved nor indeed whether

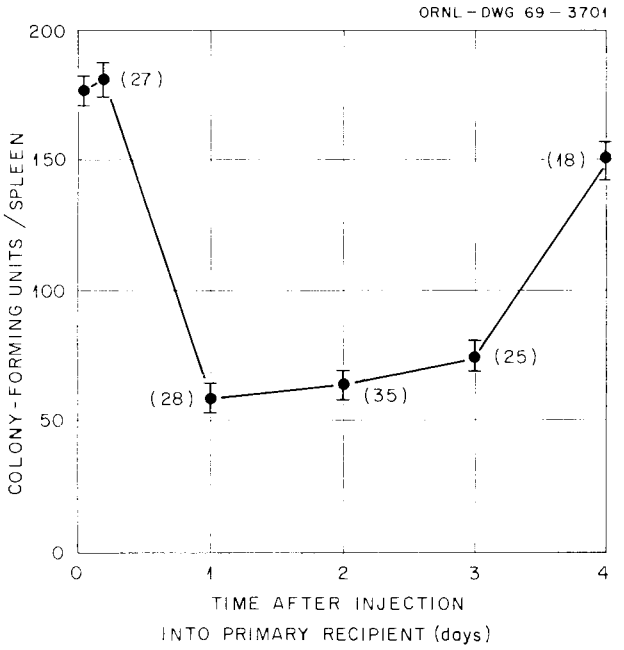

Fig. 11.9.1. Colony-Forming Cells in Spleen of Lethally Irradiated Mice Given 1 × 10^6 Syngeneic Bone Marrow Cells, Showing the Substantial Decrease in Number that Occurs Between 4 hr and One Day After Injection. Points are means calculated from the number of spleens shown in parentheses. Vertical lines are one standard error.

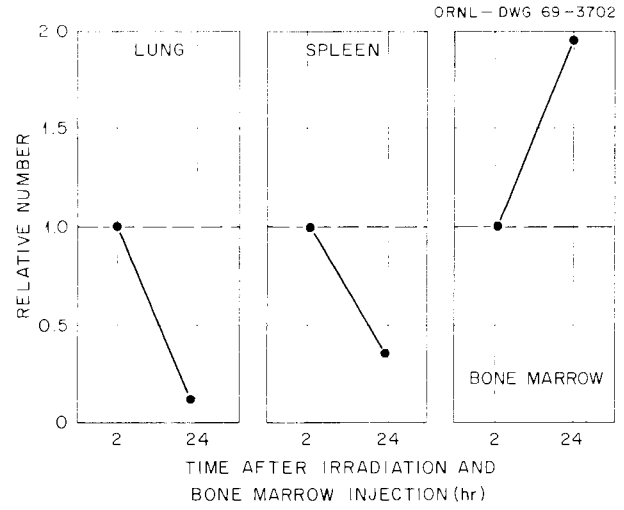

Fig. 11.9.2. Content of Colony-Forming Cells in Tissue of Lethally Irradiated Mice Given Syngeneic Bone Marrow Cells, Showing Rapid Decrease in Lung and Spleen but an Increase in the Bone Marrow. The data for each tissue were normalized to the number of colony-forming cells found at 2 hr.

it can be achieved. Experiments directed to answering this question are in progress.

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[1] The University of Tennessee Memorial Research Center; in collaboration with the Mammalian Recovery section.

[2] A. L. Kretchmar and W. R. Conover, *Biol. Div. Semiann. Progr. Rept. Dec. 31, 1967*, ORNL-4240, p. 164.

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11.10 GENERATION TIME OF SPLEEN COLONY-FORMING CELLS IN LETHALLY IRRADIATED MICE GIVEN SYNGENEIC BONE MARROW CELLS

A. L. Kretchmar W. R. Conover[1]

The model for the action of vinblastine in vivo published by Valeriote *et al.*[2] gives a theoretical basis for calculation of generation time from cell survival data.

Lethally irradiated mice were given syngeneic bone marrow cells and divided into two groups. One group was injected with vinblastine 24 hr after irradiation and bone marrow treatment. The other group was given vinblastine at 72 hr. In both groups, the drug was given as a single dose in saline by intraperitoneal injection. Within each group, animals were given a range of doses from 5 γ to 360 γ per mouse. Animals were killed 24 hr after vinblastine injection and cell suspensions made from their spleens. Aliquots of these suspensions were injected into lethally irradiated mice, which were killed nine days later, and the number of colony-forming cells in spleens of the vinblastine-treated animals was calculated from the number of spleen colonies found at nine days in the secondary recipients.

The data are summarized in Table 11.10.1, where the fractional survival of colony-forming cells is shown relative to the dose of vinblastine given.

The Valeriote model defines D_k as that threshold dose below which no cells are killed and fractional survival is 1. For doses higher than D_k, cells will be killed depending upon how long effective levels of drug are maintained in the animal and the generation time of the cells. The level of drug decays with a half-time, in mice, of 3.5 hr. If one gives multiples of D_k by single

injection, then the generation time of the cells can be estimated from the fractional survival data, since the fractional survival goes to zero for

$$D \geqslant D_k \cdot 2^a ,$$

where

$a = T_g/T_{1/2}$,

T_g = generation time of cells,

$T_{1/2}$ = 3.5 hr, the half-time for vinblastine,

D = dose of vinblastine injected,

D_k = threshold dose of vinblastine.

Data (Table 11.10.1) indicate that colony-forming cells are dividing at 24 hr after transplantation with a generation time greater than four times ($16 = 2^4$) the vinblastine half-time. This suggests that the generation time is greater than 14 hr. At 72 hr after transplantation they are dividing with a generation time of about 10 hr. The increase in vinblastine sensitivity with time after transplantation of colony-forming cells suggests that their generation time is not constant but becomes progressively shorter over the first 72 hr after transplantation.

Table 11.10.1. Survival of Colony-Forming Cells in Spleens of Primary Recipients Treated with Vinblastine After X Ray and Syngeneic Bone Marrow

Dose (D_k units)	Fractional Survival After Injection at –	
	24 hr	72 hr
2	0.45	0.42
4	0.45	0.15
8	0.11	0.04
16	0.09	0

References

[1] The University of Tennessee Memorial Research Center; in collaboration with the Mammalian Recovery section.

[2] F. A. Valeriote, W. R. Bruce, and B. E. Meeker, *Biophys. J.* **6**, 145 (1966).

11.11 EFFECT OF HYPOXIA, IRRADIATION, AND BONE MARROW TRANSPLANTATION ON ERYTHROPOIETIN PRODUCTION IN MICE

T. P. McDonald[1] R. D. Lange[1]

Preliminary studies have indicated plasma increases in erythropoietin (ESF) levels in mice following irradiation and hypoxia. ESF production was therefore studied in five groups of female $B6C3F_1$ mice after treatment with (1) hypoxia (approximately 7% O_2) produced by enclosure in silicone rubber membrane cages; (2) 450, 750, 950, and 1200 r of x ray; (3) hypoxia and x ray; (4) hypoxia, x ray, and injection with 1, 5, and 15×10^6 homologous bone marrow cells; and (5) x ray and bone marrow cells. Mice from each of the above treatment groups were bled at daily intervals for ten days, and the pooled plasma was assayed for ESF by the ^{59}Fe polycythemic mouse assay. Normal mice enclosed in cages produced about 2.5 units of ESF per milliliter of plasma after 24 hr. The circulating ESF levels then decreased to less than 1 unit/ml as the hematocrit values increased. Irradiated mice at normal O_2 levels had undetectable amounts of ESF until day 8. The ESF levels then rose sharply as hematocrits decreased, and on day 10 the mice produced about 4 units of ESF per milliliter of plasma. However, in experiments in which mice were exposed to x ray and hypoxia, distinct cyclic elevations of ESF activity were observed. Mice given bone marrow transplants after irradiation did not show the characteristic increase in ESF levels by day 10. However, markedly increased levels of ESF with obvious cyclic elevations were observed in mice enclosed in membrane cages following irradiation and bone marrow transplantation. These data indicate that transplantation of small numbers of bone marrow cells markedly alters the pattern of ESF elaboration following lethal doses of x ray.

Reference

[1] The University of Tennessee Memorial Research Center; in collaboration with the Mammalian Recovery section.

11.12 FERROCHELATASE ACTIVITY AS AN INDEX OF ERYTHROID CELL ACTIVITY IN TISSUES OF SPLEEN-SHIELDED RATS RECOVERING FROM X IRRADIATION

A. Mazur[1] L. H. Smith

Introduction. – Studies in our laboratory have demonstrated that change in ferrochelatase activity,

measured in spleen and marrow of rats treated by a variety of procedures, is a biochemical index of alteration in the erythropoietic activity of these tissues.[2] This enzyme (also called heme synthetase), which catalyzes the formation of heme from protoporphyrin and Fe^{2+}, is found in liver in the mitochondrial membrane and in the membrane of the reticulocyte but is absent from adult red cells.

Results and Conclusions. – Figure 11.12.1 illustrates the changes in ferrochelatase activity, per gram of tissue, of rats exposed to 900 r of whole-body x irradiation as well as of protected rats whose spleens were exteriorized and shielded with lead during exposure to 900 r. The unirradiated controls were subjected to a sham operation, and values for ferrochelatase activity of each tissue in the control rats were adjusted to 100 for comparison with those obtained with tissues of treated animals. None of the unprotected rats survived beyond the eighth day, whereas some of the protected rats survived at least 29 days following irradiation.

The marked decrease in spleen ferrochelatase activity of protected rats, which lasted for at least 8 days, varied in subsequent experiments, lasting sometimes only for 2 days, and recovered its enzyme activity, reaching values twice that of the normal controls, before decreasing to normal values by about the 29th day. Bone marrow activity of protected rats also fell to very low values and then increased slowly, reaching normal values by the 29th day. Liver enzyme activity of protected rats was somewhat depressed following irradiation and then rose to values about 50% above that of control rats before decreasing to normal values. The effect of irradiation on red cell ferrochelatase activity followed exactly that noted for the spleen and reflects the relative number of red cells that are actively engaged in hemoglobin synthesis.

That the change observed in ferrochelatase activity of the spleen was an index of altered metabolism of erythroid cells is demonstrated by the results shown in Table 11.12.1, which compares the ability of respiring spleen slices from protected rats to incorporate serum-bound ^{59}Fe into hemoglobin and ferritin and of ^{14}C-thymidine into total DNA, with comparable activity of an equal weight of spleen slices from control rats, the latter adjusted to values of 1.0 in each instance. On the second day following irradiation, when ferrochelatase activity of the spleen of irradiated rats is lower than that from control rats, the relative incorporation of isotopes after 1 hr of incubation was 80, 60, and 30% of that in control slices for hemoglobin, ferritin, and DNA respectively. In this series, recovery is evident in spleen slices by the seventh day following irradiation.

These results are in general agreement with the interpretation of the course of events of spleen-shielded rats following irradiation, as a result of histological

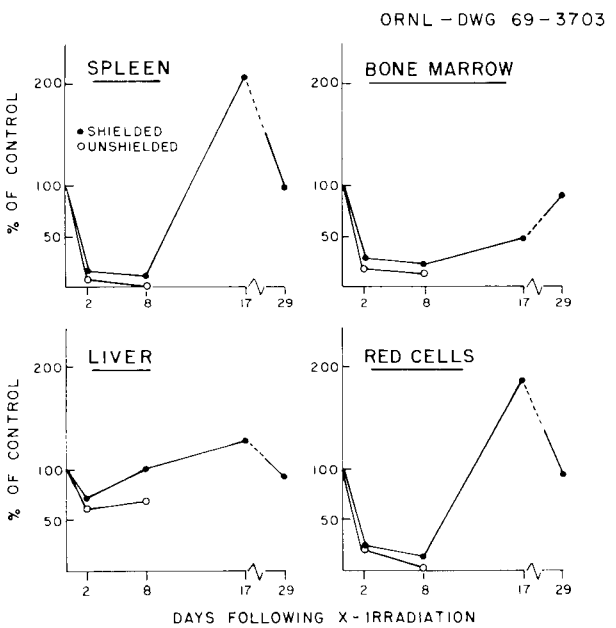

Fig. 11.12.1. Relative Changes in Tissue Ferrochelatase Activity in Control and Spleen-Shielded Rats Receiving 900 r Whole-Body Irradiation. Each point represents the average of three to six rats.

Table 11.12.1. In Vitro Activity of Spleen Tissue Slices from Spleen-Shielded and X-Irradiated Rats

Equal weights of spleen slices from normal control rats and x-irradiated spleen-shielded rats were incubated with equal quantities of serum-bound ^{59}Fe or ^{14}C-thymidine for 1 hr. In each instance the results are expressed by comparing with those of control experiments, the latter values adjusted to 1.0.

Days After Irradiation	Relative Incorporation (controls = 1.0)		
	Hb (^{59}Fe)	Ferritin (^{59}Fe)	DNA (^{14}C)
2	0.8	0.6	0.3
7	2.6	2.2	4.4
14	3.0	2.2	3.8
21	2.4	1.2	1.9

studies by many workers, and can be explained in terms of protection of stem cells in the spleen during irradiation and subsequent "seeding" of the bone marrow. No clear interpretation is yet possible for the initial decrease in ferrochelatase activity of spleens from spleen-shielded rats, unless it is due to (1) insufficient shielding for full protection or (2) the effect of blood-borne inhibitors resulting from irradiation of the whole body. In any event, the primitive stem cell would appear to be less vulnerable to the effects of this factor, irradiation or products of irradiation, than are the more developed erythroid cells engaged in heme synthesis.

The effect of irradiation on the lead-shielded spleen with respect to incorporation of iron into ferritin in respiring spleen slices follows closely the effects on hemoglobin synthesis and again suggests that ferritin accompanies erythroid cells, as we have demonstrated in the rat reticulocyte.[3]

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[1] The New York Blood Center, New York City; supported by grants from the National Institutes of Health, the American Cancer Society, and the U.S. Atomic Energy Commission; in collaboration with the Mammalian Recovery section.

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[3] A. Mazur and A. Carleton, *J. Biol. Chem.* **238**, 1817 (1963).

11.13 ^{59}Fe DISTRIBUTION IN HEMOPOIETIC TISSUE: ESTIMATE OF TOTAL BONE MARROW CELLULARITY

L. H. Smith M. L. Clayton

Introduction. – As part of our program to characterize the effects of radiation on the hemopoietic system of the mouse, we have made extensive use of ferrokinetic methods. The present report examines the distribution of injected ^{59}Fe in hemopoietic tissues of normal mice and describes a method whereby an estimate of the number of bone marrow cells in a mouse can be made.

Materials and Methods. – Female (C3H × C57BL)F_1 mice 12 to 14 weeks old were used. At intervals after intravenous injection of 1 μc of ^{59}Fe, mice were anesthetized and were perfused with saline through a lateral tail vein – retro-orbital sinus route. After extensive perfusion the mice were completely dissected, and the ^{59}Fe activity of all tissues was determined. Only ^{59}Fe distribution in bone marrow and spleen is considered in this report.

Results and Conclusions. – Table 11.13.1 shows ^{59}Fe distribution in bone marrow of different parts of the skeleton as a function of time after isotope injection. There was a rapid uptake of ^{59}Fe which reached a maximum in about 6 hr. The largest single compartment was contained in the vertebral column, which incorporated about one-third of the total ^{59}Fe detected in bone marrow.

Table 11.13.1. Distribution of ^{59}Fe in Skeletal and Splenic Hemopoietic Tissue of the Mouse

	Time After ^{59}Fe Injection (hr)[a]										Contribution[b]
	1/4		1	2	4		6		12	24	(% ± S.E.)
Vertebra (all)	4.7	6.3	8.9	10.8	15.6	12.9	18.5	15.9	15.6	10.0	10.2	34.8 ± 0.6
Femur (2)	1.4	1.7	2.4	4.3	5.0	4.3	6.8	5.9	6.0	4.1	4.0	12.0 ± 0.4
Pelvis	1.3	1.5	2.2	3.6	5.0	3.9	5.6	5.6	5.5	4.0	3.5	10.9 ± 0.4
Skull (with facial bones)	1.5	1.7	2.1	2.9	3.2	3.5	3.5	3.3	3.7	2.9	3.0	8.8 ± 0.4
Ribs and sternum	1.3	1.5	1.6	2.7	3.3	3.0	3.3	3.9	2.9	1.9	2.4	7.7 ± 0.3
Fibula, tibia, hind foot (2)	1.1	1.0	1.4	2.2	3.0	2.2	3.3	3.3	3.6	2.3	2.1	6.8 ± 0.3
Humerus (2)	0.6	0.9	1.1	2.0	2.3	2.1	3.1	3.0	2.7	1.8	1.7	5.6 ± 0.2
Scapula and clavicle (2)	0.4	0.9	0.6	0.9	1.0	1.0	1.2	1.3	1.0	0.7	0.7	2.8 ± 0.3
Mandible	0.3	0.3	0.5	0.7	0.6	0.8	1.1	0.6	0.9	0.9	1.0	2.1 ± 0.2
Radius, ulna, forefoot (2)	0.3	0.4	0.4	0.5	0.5	0.5	0.6	0.5	0.6	0.4	0.4	1.5 ± 0.1
Spleen	0.4	0.9	2.7	3.7	3.9	4.0	1.9	2.9	2.3	1.5	2.0	7.0 ± 0.9
Total	13.3	17.1	23.9	34.3	43.4	38.2	48.9	46.2	44.8	30.5	31.0	100.0

[a] Values are percentages of total ^{59}Fe counts injected.

[b] Values are percentages of ^{59}Fe counts in skeletal and splenic hemopoietic tissue.

Data in Table 11.13.1 were used to estimate the total number of bone marrow cells in the mouse by the following method:

P_f = percent ^{59}Fe uptake by two femurs

C_f = number of nucleated cells in two femurs

P_t = percent ^{59}Fe uptake by entire bone marrow

F_p = percent of total hemopoietic ^{59}Fe taken up by two femurs

$F_p = (P_f/P_t) \times 100$

Knowing C_f and F_p, the total number of cells, C_t, can be calculated from

$$F_p/100 = C_f/C_t .$$

The number of bone marrow cells in two femurs, C_f, is based on the fact that on the average we obtain 17×10^6 cells from one femur. However, since all cells cannot be flushed from a femur, 20×10^6 is thought to be a more correct value. Total hemopoietic cell number was determined for the 11 mice used in this study, and a value of $14.8 \pm 0.6 \times 10^9$ cells per kilogram body weight was obtained. The validity of the method used for these calculations is based on the assumption that ^{59}Fe uptake per unit number of cells is reasonably uniform in the hemopoietic tissues studied. In the last column of Table 11.13.1 is an estimate of the contribution of marrow in each of the skeletal parts examined.

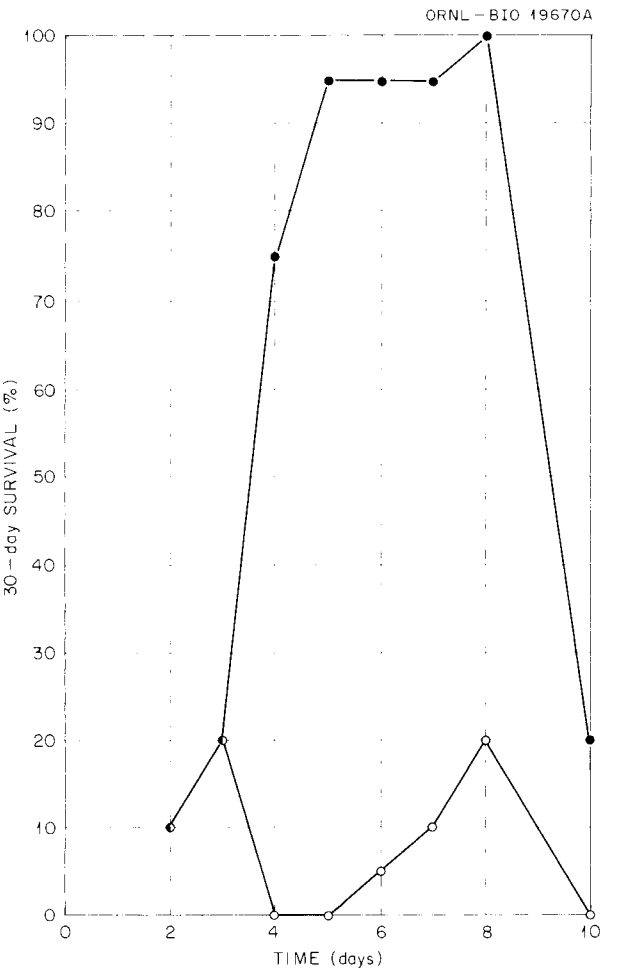

Fig. 11.14.1. Effect of Phenylhydrazine Injected Before Irradiation (800 r) on 30-day Survival of Mice. •, phenylhydrazine; ○, water. Twenty mice per point. Abscissa is time of irradiation after the third of three daily injections (1.0 mg/day) of phenylhydrazine.

11.14 PROTECTION AGAINST LETHAL X IRRADIATION BY PHENYLHYDRAZINE

L. H. Smith T. W. McKinley, Jr.

Introduction. – There is good evidence of a direct, but not necessarily causal, relationship between erythropoietic activity and radioresistance. The present report describes the effect of phenylhydrazine, an erythropoietic stimulant, on survival of x-irradiated mice.

Materials and Methods. – Male and female (C3H × C57BL)F_1 mice 12 to 15 weeks old were used. They were injected subcutaneously with PHCl (phenylhydrazine hydrochloride) in water. At various intervals after injection, the mice were exposed to 250-kvp whole-body x rays, and 30-day survivors were scored.

Results and Conclusions. – Data of Fig. 11.14.1 show that PHCl given before irradiation increases radioresistance, thereby confirming the erythropoietic data of Jacobson *et al.*[1] Tissues other than those engaged in erythropoiesis also must be involved because myelopoietic, thrombopoietic, and lymphopoietic compartments must regenerate to prevent acute radiation deaths.

The mechanism of protection is not clear, but there are several possibilities to consider. PHCl or its metabolic products may afford a chemical protection like that obtained with cysteine or AET. However, we found that PHCl given shortly before irradiation has no protective effect. Since erythrocytes which are damaged by PHCl create an acute disposal problem for the animal, hyperactivity of the RES probably occurs, thereby improving defense against infection. We are not aware, however, of evidence that a hyperactive RES

increases radioresistance. It was suggested that as a consequence of increased erythropoiesis, stem cells are triggered into cell cycle and that these cells are more radioresistant than nontriggered cells. However, our preliminary evidence, together with that of G. Hodgson (personal communication), suggests that radiosensitivity of marrow from animals given PHCl is essentially normal. Finally, PHCl may increase the number of hemopoietic stem cells by compensatory mechanisms resulting from direct stem cell damage or from consequences of erythropoietic stimulation. The increase in cell number would improve chances of survival because the number of cells surviving is a function of the number present at the time of irradiation.

Reference

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12. Experimental Hematology

Joan Wright Goodman	Chareen G. Grubbs Sarah C. Shinpock Kathryn J. Woodle[a]

[a]Student Trainee

12.1 INFLUENCE OF THYMUS CELLS ON ERYTHROPOIESIS: FURTHER STUDIES

Joan Wright Goodman Sarah G. Shinpock
Chareen G. Grubbs

Investigation of factors that modify the initial growth of hemopoietic transplants suggested that thymus may be involved in erythropoiesis. Previous studies[1] showed (1) when viable parental (P1) thymocytes were given to heavily irradiated (P1 × P2)F_1 hybrid mice along with 10^6 P1 bone marrow, a six- to eightfold increase was seen in erythropoiesis at one week; (2) the augmentation was specific, in that P2 and F_1 thymocytes did not give results comparable with those derived from P1 thymus; (3) viable F_1 thymocytes did improve growth of P1 marrow in the F_1 hybrid, but this effect was not regular and did not exceed a twofold increase; (4) when P1 thymocytes were given intraperitoneally or were heavily irradiated before intravenous administration, they were ineffective; (5) they were, however, effective when given intravenously as much as two days before or two days after the marrow inoculum; and (6) their effectiveness was thymus dose dependent in the range of 20 to 160 million cells.

The possibility that the augmentation resulted from a humoral thymic factor, analogous to that which is partly responsible for the immunological role of the thymus,[2] was investigated by implantation of Millipore diffusion chambers containing P1 thymus cells in the peritoneal cavities of F_1 hybrid mice that had been heavily irradiated and then treated with P1 bone marrow. In two experiments involving 27 (B × C3)F_1 hybrids implanted with B thymus (one organ per recipient, sliced) in Millipore diffusion chambers and 23 (B6 × D2)F_1's implanted with suspended B6 thymic cells (10^8 per recipient) in chambers, equivalent numbers of appropriate controls being included in both cases, there was no change in erythropoiesis one week after transplantation. These results suggest that if there is a thymic humoral factor influencing erythropoiesis, it is not effective at any distance from the site of its elaboration in vivo.

Because of the change of size and immunological importance of the thymus with age, it was of interest to determine whether similar changes with age occur in the influence of thymocytes on erythropoiesis. Two experiments were done, with a total of 342 (B6 × D2)F_1 males irradiated and then given 10^6 B6 bone marrow cells. Groups of 30 mice each were given (intravenously) thymus cell suspensions prepared from B6 mice 1, 5, 9, or 13 weeks old. A week later 24-hr ^{59}Fe uptake into erythrocytes and spleens was assessed, and it was found that thymus of all age groups had significantly improved (three- to eightfold) poor growth of B6 bone marrow in the hybrids. The youngest donors in both experiments were most effective, but there was not a regular decline in effectiveness with donor age.

Poor growth of parental marrow in F_1 hybrids is not a general finding but occurs in relatively few genetic combinations. We wondered, therefore, whether the augmentation of erythropoiesis produced by thymus was limited to these particular cases or would also be seen in other transplantation situations, for example, allogeneic and xenogeneic, where marrow growth is relatively poor. (B6 × D2)F_1 hybrids were irradiated

and then given marrow transplants of 10^6 A.SW (two experiments) or 10^7 rat cells (three experiments), either with or without thymus cells (syngeneic with marrow). In all five experiments significant augmentation of erythropoiesis (three- to sixfold) by thymocytes was recorded. Two other allogeneic combinations, B6 → D2 and D2 → (B × C3)F_1, were investigated, but mortality was very great among thymus recipients, and no augmentation was seen. It is probable that graft-vs-host (GVH) activity of thymocytes obscured any augmentative effect.

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12.2 AUGMENTATION OF ERYTHROPOIESIS BY THYMOCYTES: POSSIBLE ROLE OF GRAFT-vs-HOST (GVH) ACTIVITY

Joan Wright Goodman Sarah G. Shinpock

Inasmuch as thymocytes are known to be immunocompetent when given in large numbers to nonisogenic mice,[1] it was of interest to examine the possible role that GVH activity might play in bringing about augmentation of erythropoiesis. For this purpose two kinds of studies were undertaken: (1) the effect of viable Pl lymph node cells injected intravenously into irradiated F_1 hybrid mice treated with Pl bone marrow and (2) selection of thymus donor:marrow donor:host combinations such that GVH activity might be present in the absence of graft-vs-graft (GVG) activity.

The results of these investigations are shown in Table 12.2.1. Experiments A and B show that parental lymph node cells isogenic with transplanted bone marrow failed to improve erythropoiesis. Cell doses used in these studies, 4 to 32 × 10^6, were low compared with the 10^8 dose of thymus cells administered in analogous experiments. However, two earlier studies had shown that higher doses of parental lymphocytes, 32 to 100 × 10^6, could not be used because they resulted in death of 75 to 100% of the F_1 hybrid recipients.

Experiments C, D, and E show that GVH activity per se does not appear to alter poor growth of Pl marrow in F_1 hybrids. When thymus donor was allogeneic to the host (and therefore capable of GVH), (B6 × C3)F_1 in experiments C and E, but incapable of GVG because marrow was parental (B6) to it, no augmentation was seen. When marrow donor was allogeneic instead of parental to the host (experiment D), thymus cell grafts capable of GVH produced a *decrease* in erythropoiesis. Only B6D2F_1 thymus, incapable of either GVH or GVG, gave a significant though slight augmentation.

These experiments, therefore, suggest that GVH activity alone cannot improve poor growth and that the mechanism whereby thymocytes are effective must depend on factors other than immunocompetence of the cells.

Reference

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12.3 HISTOLOGIC EVALUATION OF INCREASED MARROW GROWTH RESULTING FROM THYMUS CELL TRANSPLANTATION IN PARENT-TO-F_1 HYBRID CHIMERAS

Joan Wright Goodman Chareen G. Grubbs

That parental thymocytes increase the early growth of all elements of transplanted parental (Pl) marrow in irradiated F_1 hybrid mice has been inferred from ^{59}Fe-uptake studies and spleen weight values.[1] The possibility that erythropoiesis was being preferentially stimulated at the expense of other hemopoietic pathways needed to be investigated.

Earlier experiments had shown that hybrid mice given parental marrow and thymus had macroscopically confluent splenic nodules by day 9 or 10, at which time recipients of marrow only had just a few countable colonies. It was evident that examination should be made at earlier time intervals and that spleens should be looked at microscopically.

(B × C3)F_1 hybrid mice were irradiated and given 10^6 B marrow cells with or without 10^8 viable thymocytes. Mice from the several groups in each experiment were killed at intervals from 1 to 11 days after marrow was given. Spleens were weighed and then fixed in Tellyzinsky's fluid for macroscopic examination. A longitudinal section (5 μ thick), adequately representative of the whole spleen,[2] was cut from the middle portion of each spleen and stained with hematoxylin and eosin. Colonies were identified and counted microscopically as shown in Table 12.3.1.

Table 12.2.1. Effect of Nonisogenic Lymphocytes and Thymocytes on Growth of Parental Marrow Cells in Irradiated F_1 Hybrid Mice

Experiment	Irradiated (900 r) Recipient	10^6 BM Donor	Lymph Node		Donor of 10^8 Thymus Cells	24-hr ^{59}Fe Uptake (% of Injected Dose) in RBC
			Donor	Cell Dose		
				$\times 10^6$		Mean ± S.E.
A	(B10 × B10.D2)F_1	B10	B10	32	None	0.4 ± 0.1
	(B10 × B10.D2)F_1	B10	B10	16	None	0.4 ± 0.1
	(B10 × B10.D2)F_1	B10	B10	8	None	0.3 ± 0.04
	(B10 × B10.D2)F_1	B10	B10	4	None	0.9 ± 0.1
	(B10 × B10.D2)F_1	B10	None	None	None	1.7 ± 0.2
	(B10 × B10.D2)F_1	None	None	None	None	0.3 ± 0.1
B	(B × C3)F_1	B	None	None	B	8.0 ± 0.6
	(B × C3)F_1	B	None	None	(B × C3)F_1	2.9 ± 0.3
	(B × C3)F_1	B	B	8	None	1.3 ± 0.1
	(B × C3)F_1	B	(B × C3)F_1	8	None	1.5 ± 0.1
	(B × C3)F_1	B	None	None	None	1.4 ± 0.1
	(B × C3)F_1	None	None	None	None	0.5 ± 0.1
	(B × C3)F_1	None	None	None	B	0.2 ± 0.1
	(B × C3)F_1	None	None	None	(B × C3)F_1	0.5 ± 0.04
C	(B6 × D2)F_1	B6	None	None	B6	6.3 ± 0.5
	(B6 × D2)F_1	B6	None	None	(B6 × C3)F_1	1.5 ± 0.3
	(B6 × D2)F_1	B6	None	None	None	1.0 ± 0.1
	(B6 × D2)F_1	None	None	None	None	0.3 ± 0.1
D	D2	B6	None	None	B6	0.2 ± 0.03
	D2	B6	None	None	(B6 × C3)F_1	3.8 ± 0.5
	D2	B6	None	None	(B6 × D2)F_1	11.5 ± 1.2
	D2	B6	None	None	None	7.1 ± 0.7
	D2	None	None	None	None	0.4 ± 0.1
E	(B6 × D2)F_1	B6	None	None	B6	8.2 ± 0.9
	(B6 × D2)F_1	B6	None	None	(B6 × C3)F_1	1.6 ± 0.2
	(B6 × D2)F_1	B6	None	None	(B6 × D2)F_1	2.6 ± 0.2
	(B6 × D2)F_1	B6	None	None	A.BY	0.3 ± 0.04
	(B6 × D2)F_1	B6	None	None	None	1.6 ± 0.1
	(B6 × D2)F_1	B6	None	None	None	0.2 ± 0.03

Mice that received 10^8 B thymocytes along with 10^6 B marrow cells had eight to ten colonies per spleen on day 4, whereas marrow-only recipients had just three colonies on day 5. Colonies had become confluent and therefore uncountable after day 7 in the first group, but confluency was never encountered in the marrow-only group within the period of observation in these two experiments. The relatively few colonies seen in this group at the later intervals, however, were very large.

One group of nine mice received thymocytes from baby parental strain mice (<8 to 54 hr old). These were all killed on day 6, and, as can be seen from Table 12.3.1, their total and differential colony counts did not differ substantially from those that had been treated with adult thymus. Because newborns are immunologically immature, these results suggest the augmentation of marrow growth does not depend on immunological competence of the thymus.

Table 12.3.1. Hemopoietic Colonies in Spleens of Heavily Irradiated (B × C3)F_1 Females: Effect of Thymus Cells

Treatment			Mean Number of Colonies per Spleen	Differential Count (percentage)				
BM, B	Thymus	Day[a]		Stem	Eryth.	Myelo.	Meg.	Mixed
			Experiment A					
10^6	B,10^8	4	10.1	52.7	35.1	11.0	1.2	0
		5	17.9	37.3	52.8	9.3	0.6	0
		6[b]	29.7	21.3	64.1	7.9	1.5	5.2
10^6	BC3F_1, 10^8	4	0.7	83.3	16.7	0	0	0
		5	10.6	50.6	41.0	7.3	1.1	0
		6	8.0	20.9	75.0	4.1	0	0
		7[b]	19.3	28.1	57.5	13.3	0.5	0.5
10^6	None	5[c]	3.1	35.7	64.3	0	0	0
		6	5.1	17.4	82.6	0	0	0
		7	8.7	11.6	87.2	1.2	0	0
		10	9.8	18.3	65.8	13.6	2.3	0
		11	7.7	13.1	78.4	8.5	0	0
None	None	5[c]	0.3	100	0	0	0	0
		6	0.3	0	0	0	100	0
		7	0	0	0	0	0	0
		10	0.3	0	100	0	0	0
		11	0	0	0	0	0	0
None	B, 10^8	6[c]	0.3	0	0	100	0	0
		7	1.2	0	0	100	0	0
		10	0	0	0	0	0	0
		11	1.0	0	0	100	0	0
			Experiment B					
10^6	10^8, B, adult	4[c]	8.1	43.2	39.5	16.1	1.2	0
		6	40.6	16.8	58.3	18.2	0.7	6.0
		7[b]	44.4	5.7	68.5	22.0	0.3	3.5
10^6	10^8, B, baby	6	40.2	15.4	52.8	19.9	0	11.9
10^6	None	4[c]	0.3	33.3	66.7	0	0	0
		6	7.8	20.5	75.6	2.6	1.3	0
		7	13.1	20.0	75.2	4.8	0	0
		8	9.0	14.5	83.3	2.2	0	0
		9	15.0	8.0	84.7	6.0	0	1.3
		10	8.2	21.7	73.0	4.0	0	1.3
None	None	1–10	0					
None	10^8, B, adult	7[c]	2.0	15.0	0	85.0	0	0
		8	3.5	8.6	0	91.4	0	0
		9	0.9	0	0	100	0	0
		10	0.3	0	0	100	0	0

[a]Days after cellular treatment. Groups sampled days 4, 5, 6, 7, 10, 11 in experiment A; days 1, 2, 4, 6, 8, 10 in experiment B.
[b]Comparable numbers of mice examined at later intervals but colonies confluent and not countable.
[c]Comparable numbers of mice examined at earlier intervals, but no colonies found.

Thymus from F_1 mice also was effective in increasing the numbers of colonies from B bone marrow, as shown in the second group of experiment A, although the effect was much less pronounced than that of parental thymus. Colonies were confluent on day 10 in the F_1-thymus group, days 8 and 9 having not been examined, whereas they were confluent on day 7 in recipients of parental thymus.

An occasional colony (total of 3, of three different types, from 15 mice) was seen in radiation controls of experiment A but none in experiment B. In both experiments a few colonies were found in mice that had received parental thymus but no bone marrow. In all these cases where differentiation had occurred, colonies were exclusively myelopoietic. Additional studies are necessary to determine whether or not thymocytes normally have a limited capacity for myelopoiesis and whether this capacity is manifest in an isogeneic transplantation setting.

These studies clearly show that erythropoiesis has not proceeded at the expense of myelopoiesis in recipients of thymus and marrow. A full evaluation of differential counts must await additional data, including further control groups.

References

[1] Joan Wright Goodman and Sarah G. Shinpock, *Proc. Soc. Exptl. Biol. Med.* **129**, 417 (1968).

[2] M. L. Davis *et al.*, *Proc. Soc. Exptl. Biol. Med.* **128**, 1149–53 (1968).

12.4 IMPROVED GROWTH OF MARROW TRANSPLANTS FOLLOWING PREIRRADIATION OF THE HOST

Joan Wright Goodman H. Brownell Wheeler[1]

In previous studies of the parent-to-F_1 hybrid ($P \rightarrow F_1$) poor growth phenomenon, we observed that there was improved marrow growth, assessed by measuring erythropoiesis a week after transplantation, when the host was exposed to 200 r three weeks before an exposure to 900 r followed by marrow grafting.[2] Augmented growth of marrow grafts had been observed under different experimental conditions by others.[3] The purpose of the present study was to examine several experimental parameters that influence early growth of hemopoietic cellular transplants, including (1) pretreatment x-ray dosage, (2) time interval between pretreatment and 900 r, and (3) genetic disparity between marrow donor and host.

The effect of preirradiation x-ray dosage can be seen in the first experiment of Table 12.4.1, in which parental (B6) marrow was transplanted to F_1 hybrids. At 50 and 100 r there was no effect, but at the high doses there was considerable augmentation. A second experiment with the same $P \rightarrow F_1$ combination illustrates that as the time interval between pretreatment and 900 r is increased, the effect, after going through a maximum at 9 days, drops slightly at 12 days and sharply thereafter. The last experiment illustrates that the effect of preirradiation is general, or at least is not confined to particular donor-host combinations, for augmentation is seen in $(B6 \times D2)F_1$ hybrids given 10^6 allogeneic (A.SW) cells or 4×10^4 isogeneic cells. The fact that there is improved growth of isogenic cells after preirradiation makes it unlikely that the effect in allogeneic or semi-isogeneic ($P \rightarrow F_1$) cases results from suppression of a recognition (or immune) mechanism.

Table 12.4.1. Improved Marrow Growth in $(B6 \times D2)F_1$ Males Following Preirradiation, 900 r, and Marrow Transplantation

Bone Marrow		Preirradiation		24-hr ^{59}Fe
Donor	Number of Cells	Dose (r)	Days Before 900 r	Uptake (% of injected dose) in RBC
				Mean ± S.E.
B6	10^6	0		0.9 ± 0.1
B6	10^6	50	21	0.6 ± 0.1
B6	10^6	100	21	1.1 ± 0.1
B6	10^6	200	21	1.4 ± 0.2
B6	10^6	300	21	10.0 ± 1.5
B6	10^6	400	21	8.7 ± 0.8
None	0	0	21	0.2 ± 0.02
B6	10^6	0		1.3 ± 0.1
B6	10^6	200	5	6.4 ± 0.4
B6	10^6	200	9	6.8 ± 0.5
B6	10^6	200	12	6.0 ± 0.5
B6	10^6	200	16	2.0 ± 0.1
B6	10^6	200	21	1.8 ± 0.2
B6	10^6	200	30	1.3 ± 0.1
B6	10^6	200	44	1.7 ± 0.2
None	0	0		0.3 ± 0.04
$B6D2F_1$	4×10^4	0		2.0 ± 0.2
$B6D2F_1$	4×10^4	200	5	4.4 ± 0.3
$B6D2F_1$	4×10^4	200	19	3.0 ± 0.1
A.SW	10^6	0		2.0 ± 0.2
A.SW	10^6	200	5	12.5 ± 0.9
A.SW	10^6	200	19	9.0 ± 1.5
A.SW[a]	10^6	0		20.4 ± 2.7
None	0	0		0.2 ± 0.03

[a] A.SW irradiated recipients.

References

[1] Department of Surgery, Harvard Medical School, and Director, Veterans Administration Hospital, West Roxbury, Mass.

[2] Joan Wright Goodman and H. Brownell Wheeler, *Transplantation* **6**, 173–86 (1968).

[3] N. M. Blackett and S. Hellman, *Nature* **210**, 1284 (1966).

13. Central Experimental Animal and Testing Laboratory

C. B. Richter[a,b]
M. L. Simmons[b]

Laboratory Animal Testing and Research (13.1–13.6)

C. B. Richter[a]
R. W. Tennant
D. T. Donald
J. A. Franklin
R. E. Hand, Jr.
R. L. Hendren
Carole S. King
K. R. Layman
G. L. McNabb
W. J. Miller
F. E. Myer

Animal Facilities (13.7)

J. O. Brick[a]
T. E. Whitaker
R. L. Nelson
J. R. Wells
M. R. Anderson
R. E. Cain
G. B. Carden
J. L. Crawley
H. J. Davis
R. Davis, Jr.
R. R. Deal, Jr.
C. D. Farmer
J. L. Goddard
G. M. Greene
J. L. Grey
E. Hawkins
R. Henderson
G. W. Henley
H. E. Henley
E. J. Howard, Sr.
R. L. Hudson
H. C. Huneycutt
J. W. Jarnagin
N. H. Land
V. T. McKee
J. P. McReynolds
G. E. Moore
W. E. Petty
M. Phillips
R. W. Presley
H. J. Satterfield
C. W. Schofield, Jr.
K. C. Shell
E. T. Shepard
B. E. Sise
W. D. Skeen
H. E. Smiley
R. B. Stigall
A. E. Thomas
J. E. Whittlesey
F. R. Wilkerson
O. R. Womble
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G. F. Owens[c]

[a]Dual Assignments
[b]Dr. M. L. Simmons left the Division in December, and Dr. C. B. Richter was appointed to the position vacated.
[c]Temporary

13.1 CAGE ENVIRONMENT PARAMETERS IN MICE HOUSED UNDER POLYESTER FILTERS

C. B. Richter

The influence of protective filters on mouse cage environment was tested in a Tenny environmental chamber employing electronic and analytical means. A polyester self-erecting single-piece filter, designed and developed in cooperation with a commercial firm,[1] was tested for its ability to conduct selected gases, water vapor, and heat. Conversely, it was tested for its ability to act as a biological barrier against certain pathogenic micro-organisms.

Electronic hygrometer probes were attached to cages within the environmental chamber, and hourly recordings of temperature and humidity were made over one-week intervals on a digital recorder. Data were computerized and plotted mechanically (Fig. 13.1.1). Periodic gas sampling was conducted by withdrawing cage air into standard leaks and measuring CO_2 (gas chromatograph), NH_3 (Kitagama detector), O_2 and N_2 (mass spectrometer). Peak cage CO_2 levels did not exceed 3600 ppm, as compared with environmental chamber levels, which varied between 250 and 550 ppm. The 3600 ppm is considerably below the >1% level necessary to influence mean respiratory volume in man. NH_3 levels within cages did not exceed 50 ppm; O_2 and N_2 levels remained essentially normal. Relative humidity was regularly increased about 10% above chamber levels in acceptable filters (Fig. 13.1.1), but nighttime peaks caused by increased physical activity were rapidly dissipated. Temperature within cages was influenced upward <1°F, as the filter proved to be an excellent heat conductor (Fig. 13.1.1).

Barrier efficiency was tested by placing germfree test mice in filtered cages adjacent to unfiltered cages containing mice known to be infected with certain mouse diseases. Periodically test mice were removed, destroyed, and tested for the specific diseases. Six months after beginning this experiment, the test mice remained free of these diseases. Later examination showed that disease ingress had occurred.

These experiments showed that even under maximum practical biological stress the filter cage is an effective barrier for limited periods.

Reference

[1] Charles A. Lee Associates, Inc., Knoxville, Tenn.

13.2 LABORATORY TESTING PROGRAM

C. B. Richter J. A. Franklin R. L. Hendren

In 1968 approximately 60,000 specimens were tested or examined by the Animal Testing Laboratory. Included in these are a wide variety of biological research-related specimens examined at the request of individual investigators. Many of these were sterility checks on biological materials, microbial sensitivities, bacterial counts, etc.

The numbers and types of tests conducted are summarized in Table 13.2.1. Results show that significant strides have been made by commercial producers over the years in some areas of animal production. Salmonellosis is extremely rare; pseudomonas incidence is very low. In other areas, particularly parasite control, little progress has been made. In rats the problem of chronic respiratory disease (PPLO infections) remains undiminished in most commercial production colonies. Many of these rats are unsatisfactory for any but acute or short-term experiments.

Table 13.2.1. Summary of Laboratory Animal Tests for 1968

Number of Samples	Type of Test	Number Positive
23,045	Fecal material	*Salmonella* 2, *Pseudomonas* 297
887	Autopsies	
107	Blood *Rickettsia*	None
363	Skin grafts	28 rejects
6,396	Object samples	*Pseudomonas* 181
1,564	Parasites exam	775
1,882	Complete animal exams (monitoring programs)	See text
1,895	Free chlorine	
~21,000	Parasite treatment	
876	Miscellaneous tests	

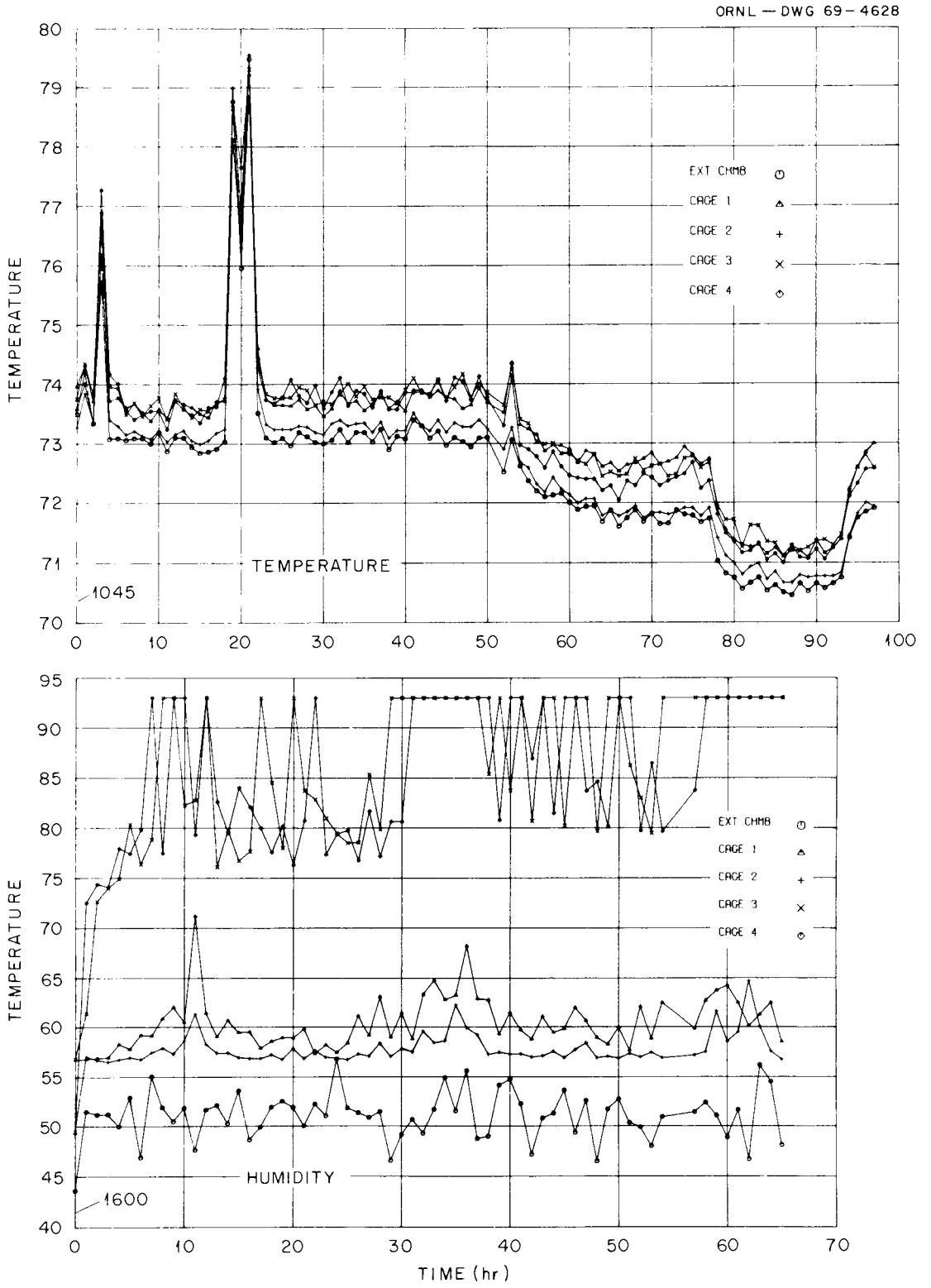

Fig. 13.1.1. Failure of Two Filters to Dissipate Cage Humidity While Levels in Two Others Remained Within Acceptable Limits. All filters were ready conductors of heat.

Monitoring programs established to examine internal barrier breeding colonies have shown excellent results. Endemic animal disease problems can be controlled by exercising reasonable protective measures. Continuing testing programs aim at producing better understanding of control measures and requisite levels of control.

A primary concern continues to center around spontaneous disease outbreaks in the animal colonies. During the year major effort was expended in investigating acute respiratory disease in DBA/2 mice. The source of the disease, type, and cause were determined, and control was established.

13.3 MORPHOLOGY STUDIES OF PLEUROPNEUMONIA-LIKE ORGANISMS (PPLO) IN VIVO

C. B. Richter J. A. Franklin Carole S. King

Little is known of the method of replication of PPLO in spite of the fact that they have been cultured in vitro for many years. Electron microscopic studies on PPLO grown on artificial media have shown several possibilities, including budding of PPLO cells, beading of filamentous forms, or fragmentation of large protoplasmic forms.[1] Numerous observations have been

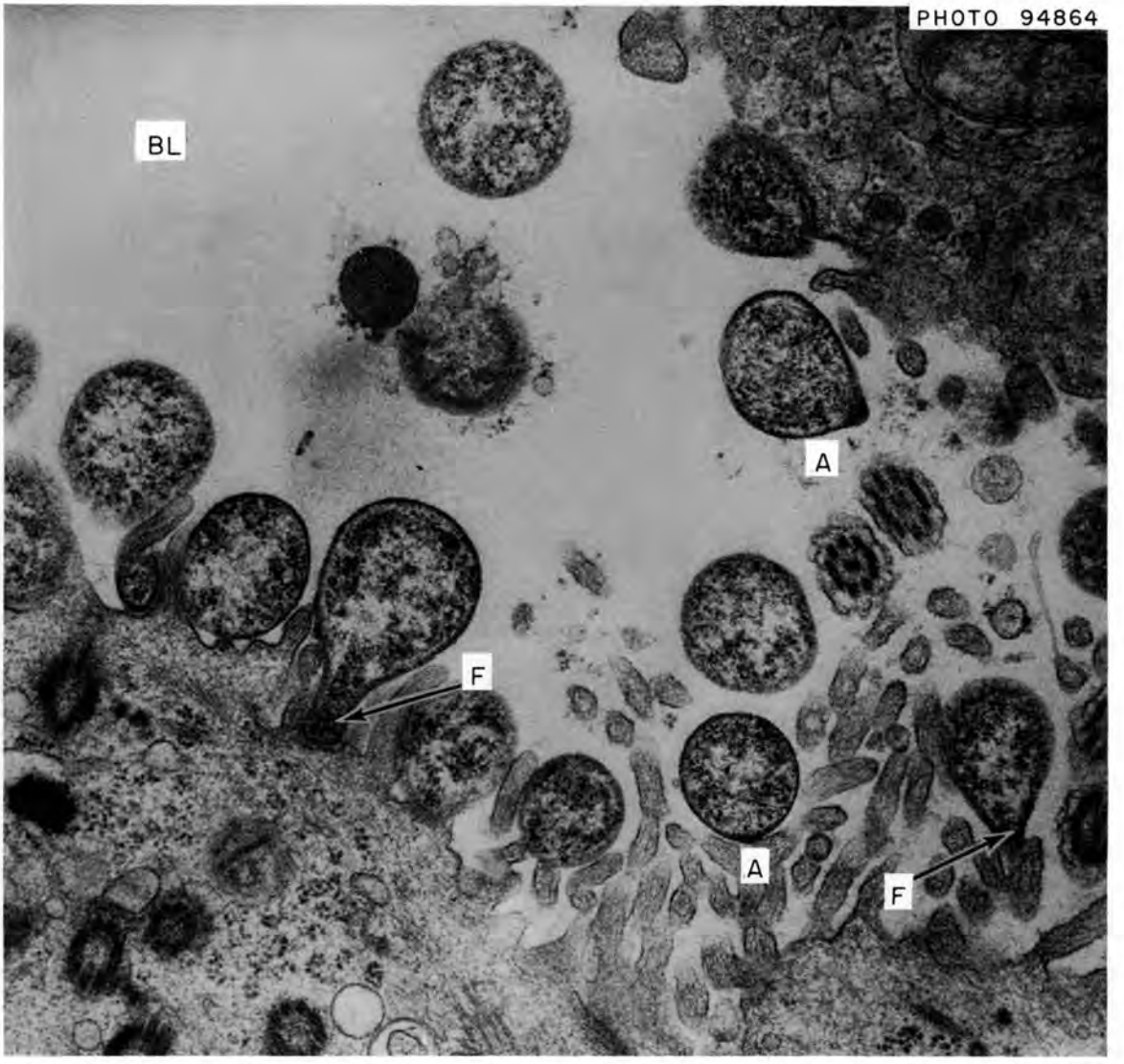

Fig. 13.3.1. Bronchial Epithelium Experimental Infection. Two PPLO show "Pseudopodia" (F); two show distinct attachment sites (A). BL = bronchial lumen. 36,000X.

made on PPLO-like organisms in tissues, but few studies have been conducted on known PPLO-infected tissues, and these have added little to knowledge of replication or physical relationship to host cells for metabolic exchange.

We established PPLO as the cause of an outbreak of respiratory disease among DBA/2 Jax mice which was characterized by a high incidence of bronchial squamous metaplasia. Electron microscope studies on infected bronchi have shown previously unreported host-cell–parasite relationships (Fig. 13.3.1). The sequence of events preceding and following these relationships is not clear. However, direct contact appears to occur with the host cell in such a manner that PPLO protoplasm may come into contact with cellular cytoplasm; this remains to be proven. Contact has been observed to occur on microvilli as well as the cell wall proper, and, where crowding keeps the organisms from close cell proximity, long ribosome-rich "pseudopodia" extending from the PPLO maintain contact. These structures, as well as smaller contact areas, are noticeably electron dense and multilayered, sometimes having as many as seven alternate dense and light layers.

Relatively "pure" cultures of PPLO "cell" forms were often seen in infected tissue (Fig. 13.3.1). Alternatively, a variety of forms similar to those described in in vitro studies[1] were seen. It is not certain as yet whether these represent different serotypes or different forms of the same organism.

PPLO infections in mouse lungs are long enduring and cause considerable morphologic damage to lung structure. Distribution of metaplastic change in bronchial epithelium occurs in scattered clusters and varies widely in intensity. Future objectives include the use of PPLO as a model for biological cocarcinogenesis.

Reference

[1] C. H. Domermuth *et al., J. Bacteriol.* 88, 727 (1964).

13.4 STUDIES ON ADOPTIVE IMMUNIZATION AGAINST LEUKEMIA IN MICE

R. W. Tennant C. B. Richter

Virtually all strains of mice examined, even though showing a low incidence of spontaneous leukemia, have revealed particles which at least morphologically resemble the known leukemia viruses. A body of evidence supports the idea that the high incidence of thymic lymphoma in AKR mice involves a state of immunological tolerance or hyporesponsiveness to a virus. This virus, immunologically similar to the agent isolated by Ludwik Gross, is the only well-defined virus associated with "natural" leukemia.

We are attempting to determine if *allogenic* cells from mice apparently free of this virus, but which have been immunized against the AKR agent, can provide protection against leukemia.

The first experiment initially involved testing the tolerance induced in AKR mice by neonatal injection of C3H spleen cells. Solid tolerance was established, and recipients of subsequent C3H skin grafts maintained them at least 11 months. In the meantime an extract was prepared from the spleens and thymuses of leukemic AKR mice and was injected into adult C3H mice at three biweekly intervals. Suspensions of spleen cells from these "immunized" and from normal C3H mice were prepared, and 100×10^6 cells were inoculated into the C3H-tolerant AKR recipients. These animals were then bred, and their progeny were injected with 30×10^6 normal or "immune" C3H spleen cells, within 24 hr post partum. The 50% leukemia mortality points were about $10\frac{1}{2}$ months after birth for the untreated and normal cell groups and $12\frac{1}{2}$ months for animals receiving "immune" cells. By 15 months after birth, control and normal cell groups exhibited 72 and 78% leukemia mortality, while 54% of the mice receiving "immune" cells had died with leukemia.

Other experiments have been initiated using as the immunogen: (1) live Gross leukemia virus grown in cell culture and (2) formalinized AKR leukemic spleen and thymus cells.

Techniques are being developed to facilitate leukemia virus isolation to determine the effect of adoptive immunization on the virus as well as the emergence of leukemia cells. In order to better define the experimental system, a leukemogenic system with a shorter latent period is being developed, but the object of the study remains to abrogate spontaneous leukemia.

13.5 CELL DESTRUCTIVE EVENTS AND THE EFFECT OF CELL STATE IN THE REPLICATION OF KILHAM RAT VIRUS (KRV)

R. W. Tennant K. R. Layman R. E. Hand, Jr.

In addition to being one of the smallest DNA viruses known (180 A), KRV is remarkable for the variety of pathologic changes which it can induce. While adult rats are infected naturally, the effects of the virus are minimal. However, infection of embryos or neonates can result in abnormalities of growth and development, making KRV one of the few viruses useful for studying birth defects.

We have attempted to use rat embryo cell cultures as a model for studying the replication and effects of KRV in vivo. Techniques used in the experiments to be reported included analysis of cell division and chromosomes, uv irradiation, and simultaneous fluorescent antibody and autoradiographic techniques.

In the following experiments we wished to determine the basis for cell injury. Cells were treated with colchicine at intervals after infection to determine the effects of KRV on both cell division and chromosomes. Mitotic activity was almost completely suppressed compared with that in mock-infected controls. However, the cells did not become necrotic until after progeny virus was formed, so there may have been a combination of events, culminating in virus release, that ultimately killed the cells. Infection of stationary or actively dividing cultures did not reveal any effects on chromosomes other than random breaks, seen with the same frequency in control cells. Therefore it does not appear that KRV teratogenesis has its basis in virus-induced chromosome damage affecting cell progeny. Rather, the effects of KRV are probably the direct result of cell killing.

Since the virus is apparently inhibitory to mitosis within 2 hr after infection, it is of interest to determine if a functional virus genome is required for mitostatic activity. KRV which was partially purified and inactivated by uv irradiation also inhibited cell division, and experiments are now in progress to determine if virus capsid protein alone is inhibitory.

While we have shown that KRV prevents mitosis in cell cultures, Margolis and Kilham[1] have presented evidence, obtained from a study of certain birth defects, that the virus has a predilection for actively multiplying cells. Hence, we attempted to determine whether active cell DNA synthesis influenced KRV replication. These experiments involved partial synchronization of cultures with fresh medium, labeling of cells in DNA synthesis with ^{3}H TdR prior to infection, and determination of virus protein synthesis with fluorescent antibody. Simultaneous analysis of cells by autoradiography and fluorescent microscopy yielded information shown in Table 13.5.1. These results show that viral protein synthesis occurred most frequently in cells engaged in DNA synthesis and that some event in the cell cycle, probably associated with DNA synthesis, affects susceptibility to the virus.

These results suggest that the teratogenic effects of KRV may be due to a selective attack on dividing cells. Further, while susceptibility to virus infection has been shown in some cases to be dependent upon cell surface receptors, our work indicates that there may also be a metabolic basis for cell susceptibility.

Reference

[1] G. Margolis and L. Kilham, *National Institute of Neurological Diseases and Blindness Monograph No. 2,* pp. 361–67, 1965.

Table 13.5.1. Viral Protein Synthesis as a Function of Cell DNA Synthesis

Group	Virus	FA Cells[a]		^{3}H-Labeled Cells[b]		FA Cells with ^{3}H[c]		FA Cells Without ^{3}H[d]		Mean Counts per Minute of ^{3}H per Microgram of Protein
		Pos./Total	%	Pos./Total	%	Pos./Total	%	Pos./Total	%	
A, infected prior to medium change	+	41/1022	4	724/1778	41	15/704	2	1/704	0.1	
	–			588/1363	43					823
				Mean = 42						
B, infected 10 hr after medium change	+	209/1161	18	887/1699	52	110/879	13	7/879	0.7	
	–			1278/1840	69					1484
				Mean = 61						
C, infected 24 hr after medium change	+	61/925	7	557/1601	35	73/807	9	2/807	0.2	
	–			874/2002	44					869
				Mean = 40						

[a] Fluorescent (FA) cells counted prior to preparation as radioautographs. Data for uninfected cultures are not shown, since no fluorescent nuclei were ever seen in the control cultures, though thousands of cells were examined.

[b] Autoradiographic count of cells labeled with ^{3}H TdR.

[c] Fluorescent cells counted after autoradiography; cells with ^{3}H label.

[d] Fluorescent cells counted after autoradiography; cells with no ^{3}H label.

13.6 ANTIBODY MONITORING FOR LABORATORY ANIMAL VIRUSES

R. W. Tennant K. R. Layman

Procedures developed previously for detection of antibody against certain murine viruses have been used to monitor mice of various ecological and experimental categories in the Biology Division. Information yielded by these tests is applied to several areas of interest. Mice in the viral Cocarcinogenesis Program are sampled and tested at intervals to ensure that barriers against intercurrent viral infections are maintained. To date, 1330 sera from this experiment have been tested for presence of antibody to the following viruses: pneumonia virus of mice (PVM), Sendai (pneumonitis), K (newborn pneumonitis), encephalomyelitis, polyoma, reovirus, mouse adenovirus, and hepatitis viruses. In addition, tests have recently been perfected for detection of lymphocytic choriomeningitis (LCM) and minute virus of mice (MVM). Test results are used also in barrier-breeder colonies, such as those supplying animals for the viral Cocarcinogenesis and MAN Programs, and in those colonies making animals available to other investigators. Positive results, where practicable, are used to implement the "test and sacrifice" procedure to maintain the colonies free of infections. A total of 1521 mice from barrier colonies have been tested, in addition to 146 germfree mice.

Tests for various investigators, special projects, and outside sources have involved 1630 mice. A large percentage of these tests involved experiments designed to test the efficacy of techniques for minimizing the effort and cost of rearing and maintaining conventional mice free of infectious agents. Results of these experiments were reported.[1] In addition, tests have been performed on 240 sera from rabbits, hamsters, and rats. The latter involve tests for Kilham rat virus and H-1 (Toolan) virus.

Techniques are now being perfected for detection of murine leukemia and sarcoma viruses.

Future goals of this approach to laboratory animal health are to arrive at relatively simple and inexpensive tests and techniques which can ensure experimental animals that yield consistent results in which investigators can have great confidence.

Reference

[1] M. L. Simmons *et al., Proc. Soc. Exptl. Biol. Med.* **126**, 830–37 (1967).

13.7 POSTIRRADIATION DEATH PATTERNS OBSERVED IN THREE GROUPS OF MICE: (1) SPECIFIC PATHOGEN FREE, (2) SEMICONVENTIONAL, AND (3) CONVENTIONAL

J. O. Brick M. L. Davis[1] J. A. Franklin

As pointed out by Miller,[2] postirradiation bacteremias are frequently caused by micro-organisms belonging to the animals' enteric flora (*Pseudomonas, Escherichia,* and *Paracolobactrum*). Recent studies in this laboratory indicated that pigmented *Pseudomonas* as well as *Streptococcus* are involved in early postirradiation death of mice. From these data, it is apparent that certain mice (germfree, defined, or pathogen free) will exhibit quite varied death patterns, with the same dose of irradiation, due to the presence or absence of specific micro-organisms.

Recently we observed 100% (150/150) mortality in less than eight days in a group of mice receiving 900 r of whole-body irradiation. These B6C3F1 hybrids had been removed from filter top and transferred to a conventional environment seven days prior to irradiation.

As a result of this observation, the response of three groups of mice were compared following a dose of 900 r of total-body irradiation. All mice were female B6C3F1 hybrids (C57BL6 ♀ × C3H ♂) which came originally from a pathogen-free barrier utilizing filter tops on all cages. The three groups were as follows:

Pathogen-Free (P-F). – The mice in this group were taken directly from the barrier to the conventional facility following irradiation. However, to maintain them essentially pathogen free, they remained in filter top cages throughout the duration of the study.

Semiconventional. – The mice of this group were removed from filter top cages and moved to a conventional facility six days prior to irradiation. These animals then remained without filter tops in the conventional environment for the remainder of the experiment. The short period prior to irradiation without filter tops allowed a change in the bacterial flora. None of the mice in this group died during this transition period.

Conventional. – This group of mice had been removed from filter tops and moved into the conventional facility 28 days prior to irradiation. They subsequently remained in the conventional environment without filter tops for the duration of the experiment.

Results. – As can be seen in Table 13.7.1, there was 100% (20/20) mortality in the semiconventional group between day 3 and day 7, whereas the first death

Table 13.7.1. Postirradiation Death Pattern

Group	Number of Mice	Number Dead	Death Loss
Pathogen free	20	18/20	Died between 11th and 22d postirradiation days
Semiconventional	20	20[a]/20	All dead between 3d and 7th postirradiation days
Conventional	54	45/54	Died between 10th and 23d postirradiation day

[a]Five morbid mice sacrificed on day 3 for bacterial culture.

occurred in the conventional group on day 10 and in the P-F group on day 11. No deaths occurred in either of these groups after day 23. Both the P-F and conventional groups exhibited an expected postirradiation death pattern for this dose rate.

From the semiconventional group, five morbid animals were sacrificed on day 3 for bacterial culturing. The results obtained did not indicate any one single organism to be involved. Furthermore, two of these mice and several mice from the original groups of 150 did not exhibit bacteremias at all. Specifically, pigmented *Pseudomonas* and *Streptococcus* sp. were not the cause of death in these animals.

The only gross pathology noted in all of the animals autopsied was a thick mucoid material in the dilated thin-walled intestinal tract. The exact cause of the early death has not been established. Additional work of this type is planned with specific-pathogen-free mice in an effort to determine the cause of early death in nonbacteremic irradiated mice.

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14. Radiation Immunology

Takashi Makinodan

Cytokinetics and Cytomorphology

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Reticuloendothelial and Microbial Systems

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Molecular Systems

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14.1 MODEL ANALYSIS OF GROWTH PATTERNS OF ANTIBODY-FORMING CELL POPULATIONS

Takashi Makinodan
Toshihiko Sado
D. L. Groves
Gerald Price[1]

In recent years there have been several comprehensive reviews, symposia, and workshops covering the subject of the growth of antibody-forming cell populations, but critical evaluation of models has been lacking. We have therefore formulated and evaluated several models because we feel that this is the best approach at this stage of development. Based on certain considerations, three major models were proposed, each composed of three subgroups, for a total of nine models. They are as follows:

A-1: Single recruitment of nondividing cells
A-2: Single recruitment of cells dividing synchronously
A-3: Single recruitment of cells dividing asynchronously
B-1: Nonrandom multiple recruitment of nondividing cells
B-2: Nonrandom multiple recruitment of cells dividing synchronously
B-3: Nonrandom multiple recruitment of cells dividing asynchronously
C-1: Random multiple recruitment of nondividing cells
C-2: Random multiple recruitment of cells dividing synchronously
C-3: Random multiple recruitment of cells dividing asynchronously

No single model should be expected to adequately explain all in vitro and in vivo systems, but the models

which may be representative of a particular system should be recognized. This information is critical to the ultimate goal – an understanding of the control mechanisms operating in a "natural system." In the comparison of experimental data with these models, it became clear that there is a need for reevaluation of the kinetics of the immune response for nearly all systems, with assessment of antibody-forming cells at intervals much shorter than the population doubling time.

There is an exception, however, in which the kinetic data are sufficient to discriminate between the various growth models. Perkins et al.[2] of this laboratory have obtained evidence of considerable synchrony in the early primary response occurring in the spleen of intact mice. Their data fit model B-2 above, with recruitment occurring at intervals equal to the generation time of the antibody-forming cells.

Further advancement of our knowledge concerning the growth of the immune response will require investigation of the homeostatic mechanisms controlling differentiation, emigration, death, and dedifferentiation.

References

[1] Student at the UT–Oak Ridge Graduate School of Biomedical Sciences.

[2] E. H. Perkins, T. Sado, and T. Makinodan, *RES – J. Reticuloendothelial Soc.* 5, 11 (1968).

14.2 RECRUITMENT AND PROLIFERATION OF IMMUNOCOMPETENT CELLS DURING THE LOG PHASE OF THE PRIMARY IMMUNE RESPONSE

E. H. Perkins Toshihiko Sado
Takashi Makinodan

Studies were carried out to elucidate the cellular events responsible for the exponential increase in the number of antibody-forming cells (log phase of immune response) by assessing the contributing roles of cellular proliferation and recruitment (enlistment of non-antibody-forming cells to antibody-forming cells). With regard to the latter role we were interested in learning whether a single or a series (multiple) recruitment is involved and, in the case of multiple recruitment, whether it is occurring randomly or nonrandomly. In order to obtain this information, a detailed kinetic study of the log phase of the immune response was carried out in a straightforward manner by assessing the number of hemolytic plaque-forming cells (PFC) in the spleen of intact mice at 2-hr intervals during the first 96 hr after stimulation with sheep red blood cells. The role of cellular proliferation in the development of PFC was analyzed autoradiographically and by using vinblastin, a mitotic inhibitor.

It was found that (1) antigen-induced cellular proliferation began about 12 hr after antigen injection, (2) PFC began to appear at a level significantly above background following a log phase of about 24 hr, (3) most, if not all, PFC during the lag phase of the response are themselves proliferating or are progeny of proliferating precursor cells, (4) the number of PFC increased nonrandomly in a staircase manner, suggesting a considerable degree of synchronous growth, and (5) there was a series of recruitment occurring nonrandomly and in phase.

These findings establish the essential and dominant role of cellular proliferation during the log phase of the primary response. It therefore seems clear that, regardless of whether antibody-forming cells are the "7S-secreting cells" characteristic of the secondary response or the "19S-secreting cells" characteristic of the early primary response, cellular proliferation is primarily responsible for their increase following antigenic stimulation. Of greater importance is the observation that multiple recruitment is occurring in a nonrandom manner. Therefore standard cellular kinetic indices, which have been used to analyze the results of such studies in the past and which have, as a primary premise, the assumption that growth is entirely random, cannot be used and necessitate reevaluation of many of the estimates of kinetic indices deduced by others.

14.3 KINETIC STUDIES OF THE GROWTH OF ANTIBODY-FORMING CELL POPULATION IN DIFFUSION CHAMBER CULTURES

Toshihiko Sado E. H. Perkins
J. M. Ellis

Recently we[1] have shown that in spleen of the intact mice undergoing primary antibody response, growth of antibody-forming cell population is not entirely random: rather, a degree of synchronous expansion exists. Furthermore, the data also show that there is a series of recruitment of functional antibody-forming cells from nonfunctional progenitors and that the recruitment size increases following the initial recruitment of antibody-forming cells. Because the spleen is an open system, there is no way of controlling the number of progenitor as well as antibody-forming cells that migrate in and out of the spleen, and therefore it was not possible to determine whether these recruits are derived from cells inside or outside the spleen. This necessitated studies in a closed system. A similar type

of experiment was therefore performed in diffusion chambers by culturing 24 X 10^6 dispersed spleen cells from previously immunized C31 F_1 mice together with 2.4 X 10^6 sheep erythrocytes. Direct and indirect plaque-forming cells (PFC) were assessed at various time intervals after culture.[2] It was found that after a latent period of about two days, direct and indirect PFC increased with doubling times of ~6 and ~4 hr, respectively, between days 2 and 4, and doubling times of ~48 and ~24 hr, respectively, between days 4 and 6. During the next few weeks the number of both direct and indirect PFC decreased with a half-time ($T_{1/2}$) of about five days. To determine if this logarithmic growth is random (linear) or nonrandom (stepwide increase) in nature, an experiment was conducted in which chambers were sampled every 2 hr for ~40 hr, starting 48 hr after culture. The result obtained with indirect PFC is shown in Fig. 14.3.1. In spite of the fairly large scattering of individual samples, the data clearly indicate that there were at least three, possibly four, stages of stepwise expansion between 60 and 82 hr. The expansion during the first two stages was dramatic, that is, 15-fold and 47-fold, respectively, suggesting massive recruitment of nonfunctional progenitors.

Autoradiographic studies of antibody-forming cells sampled from cultures which received five injections of ^{3}H-thymidine (5 μc/injection) between 24 and 48 hr (every 6 hr) indicate that most, if not all, of the antibody-forming cells observed during the early log phase are derived from proliferating nonfunctional precursors. It was also shown that as many as 50% of both direct and indirect PFC could be labeled shortly after a single pulse exposure to ^{3}H-thymidine.

Cytological studies on the rate of metaphase accumulation after administration of a mitotic inhibitor, colchicine, during various times after culture indicated an abrupt shift from slowly proliferating to rapidly proliferating population around 48 to 50 hr after culture. Careful study on the rate of accumulation of metaphase of blast cells indicated a nonrandom distribution of these cells throughout the cell cycle, that is, there is some degree of synchrony among proliferating precursors of antibody-forming cells. Thus these studies strongly suggest that antigen stimulates the proliferation of nonfunctional resting precursors in a nonrandom manner and that these cells transform into functional antibody-forming cells rather synchronously and these latter cells undergo further divisions. Further studies using this culture system may give us more insight into the factors which control such synchronous growth and expansion of antibody-forming cells.

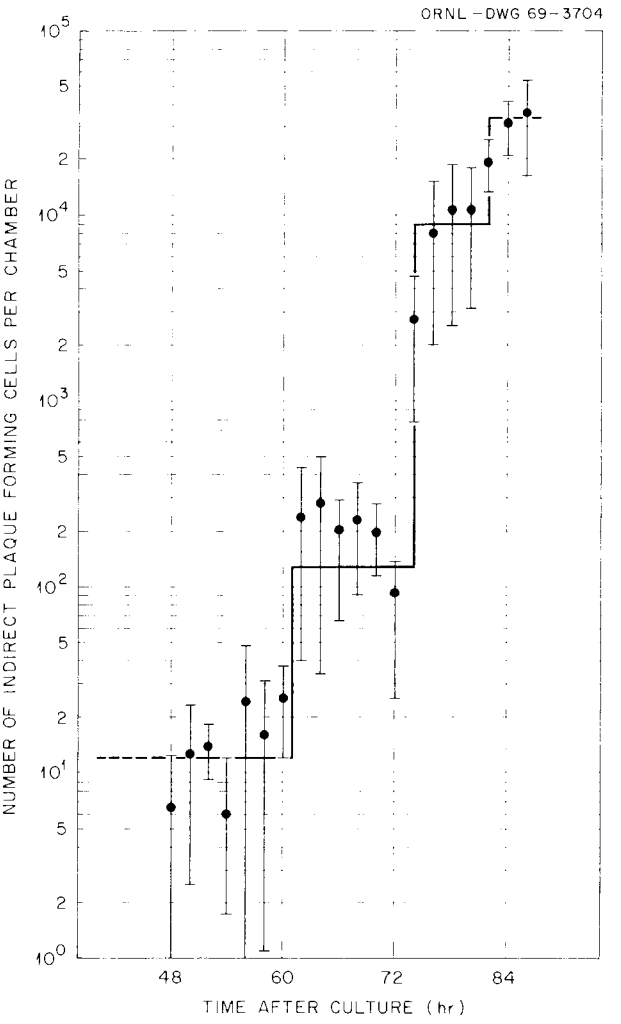

Fig. 14.3.1. Growth Pattern of Indirect Plaque-Forming Cell Population in Diffusion Chamber Cultures.

References

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[2] T. Sado, *Intern. J. Radiation Biol.* (1969) (in press).

14.4 INTERACTION OF CELL TYPES IN THE PRODUCTION OF ANTIBODY-FORMING CELLS

D. L. Groves W. E. Lever[1]
Takashi Makinodan

The primary immune response of C31F_1 mouse spleen cells to sheep erythrocytes was assessed in cell impermeable diffusion chambers by the enumeration of

antibody-forming cells [plaque-forming cells (PFC)] using the classical Jerne technique. The frequency of the presumed precursors of these PFC in the spleen cell population was determined by the limiting dilution method. Based on 231 samples, an estimate of 0.5 ± 0.1 (95% confidence limit) precursor cell per million spleen cells was obtained.[2] The shape of the limiting dilution curve indicated that the presence of only a single cell of a specific type was sufficient for a positive response or that, if two or more cells were required, only one cell type was limiting.

Dose-response studies were made, showing the relationship between spleen cell dose and the maximum number of PFC generated. A biphasic logarithmic dose-response curve was obtained, similar to that described by Bosma *et al.*[3] for a cell transfer system. At low spleen cell doses a slope of 2 was observed, but at higher doses there was an abrupt transition to a slope of 1. The slope of 2 is consistent with the interpretation that the number of PFC produced is dependent on the interaction of two cells. The transition from a slope of 2 to a slope of 1 was interpreted to mean that there is an upper limit to the degree of interaction between the two cells. That is, at higher doses, saturated interaction complexes represent the major class of immunologically competent units.

Considering the limiting dilution data and the dose-response data together, it appears that one cell may be limiting in terms of whether or not a response occurs, but that two cells may be limiting in terms of the magnitude of the response (i.e., the number of interactions).

The interpretation of these data has led to the proposal of a stochastic model for the production of antibody-forming cells based on a two-cell interaction system.[4] The model assumes that an antigen-reactive cell (ARC), having multiple interaction sites, binds precursor cells (PC) and causes their transformation into antibody-forming cells (AFC). These newly formed AFC undergo a fixed number of divisions, yielding a population of AFC. The actual number of PC which interact with an individual ARC is a function of the number of interaction sites on an ARC and the spleen cell dose.

The major requirement for the model is that it explain and reproduce the experimentally observed biphasic dose-response curve. A rigorous development of the model has been made in terms of deriving a probability distribution function for the number of PC transformed and an expression for the expected number of AFC generated. A general equation describing the biphasic dose-response curve and giving the expected number of AFC produced at a given dose is

$$E(A) = Sp\phi N_0^{2\nu} ,$$

where $E(A)$ is the expected number of AFC produced at spleen cell dose N_0, S is the number of interaction sites per ARC, p is the probability of a site on an ARC interacting with a PC at dose N_0, ϕ is the frequency of occurrence of ARC in the population, and ν is the mean number of divisions of a transformed PC. When all of the sites per ARC have not interacted with PC, as is the case for doses below the inflection point in the dose-response curve, p is less than 1 and the relationship between $E(A)$ and N_0 exhibits a slope of 2. This is because p is a linear function of dose below the inflection point. For doses above the inflection point, p is 1 and the relationship exhibits a slope of 1.

While this model is able to make several predictions which have been confirmed by experiments of others, it is most probably an oversimplification of the actual cellular events leading to the generation of antibody-forming cells. Nevertheless, it may be useful in stimulating new ideas concerning the significance of cellular interaction in the immune response and other differentiating systems.

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14.5 PRODUCTION OF HEMOLYTIC ANTIBODY-FORMING CELLS UNDER CONDITIONS OF ANTIGEN COMPETITION

J. F. Albright T. F. Omer

Previous studies, performed under suitable experimental conditions, have shown that antibody response to a given test antigen may be almost completely inhibited by a preceding injection of a non-cross-reacting antigen. Our results suggested that the two antigens were competing for a limited number of cells of a type essential for initiation of antibody production. Direct proof, however, was lacking, and thus more evidence was sought by quantitative assessment of antibody-forming cells during antigen competition.

Data presented here support this conclusion and in addition reveal preliminary information about the nature of antigen-stimulable cell units.

Recipient $C31F_1$ mice were irradiated with 850 r and injected with varying numbers of syngeneic donor cells. In experiments concerned with competition of antigens the competing antigen, horse erythrocytes (Hrbc), was injected together with the donor spleen cells. In reference controls no competing antigen was administered. Three days after injection of donor cells the recipient mice received the test antigen, 2×10^9 sheep erythrocytes (Srbc). The production of hemolytic antibody-forming cells was followed. The results were evaluated at the peak of hemolytic plaque-forming cells (PFC) produced by varying donor cell inocula. Both the plot of data for positive, reference controls and that for competing antigens show a slope of ~1. Comparison of the lines reveals that the competing antigen inhibits 70% of the yield of PFC by a given donor cell inoculum. Additional results, concerned with production of PFC as a function of donor cell inocula, were derived from experiments in which the donor cells were stimulated with Srbc at the time of transfer into recipients.[1] This curve is characterized by one limb, covering lesser cell inocula, that has a slope of ~2 and an upper limb with a slope of ~1. This curve is in sharp contrast to the one that was obtained when a three-day interval separated the injection of donor spleen cells and administration of Srbc.

The extent to which the competing antigen inhibits antibody response to the test antigen is not as great in the present experiments as was found in previous studies where antibody titer was evaluated as an index of functional cells. Nevertheless, these data indicate that ~70% of the antigen-stimulable cellular units are susceptible to stimulation with either antigen. There is an intriguing difference between the two positive reference curves. The biphasic curve was obtained when antigen was injected into recipient mice at the time of donor spleen cell injection.[1] This curve has been interpreted[2] as indicating required interaction of two different types of cells in order to form immunologically functional units. The monophasic reference curve was obtained when a three-day interval separated the injection of donor spleen cells and antigen. This curve suggests that during the three-day interval, interaction of the two types of cells occurred in the absence of antigen stimulation to produce functional units. There may be an active process in the spleen, dependent upon organized histoarchitecture, for selective localization of types of cells.

References

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[2] D. L. Groves, W. E. Lever, and T. Makinodan, *Nature* (in press).

14.6 IMPAIRMENT OF PROLIFERATION AND DIFFERENTIATION OF IMMUNOCOMPETENT CELLS IN MICE OF A HIGH LEUKEMIC STRAIN

E. H. Perkins A. C. Upton[1]
Takashi Makinodan Charlene A. Seibert
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AKR/J (AKR) mice exhibit a high incidence of age-dependent spontaneous thymic leukemia, whereas in C3HeB/FeJ (C3H) mice the disease rarely develops. Our studies indicated that the immune competence of young adult, three-month-old AKR, C3H, and (C3H × AKR)F_1 hybrid mice was comparable when assessed in terms of number of "direct" and "indirect" hemolysin plaque-forming cells (PFC) in the spleen following injection of 10^9 sheep RBC. At 12 months of age the immune competence of C3H mice was comparable with, if not slightly superior to, that of the three-month-old mice. However, ≥9-month-old AKR mice exhibited a significantly depressed immune capacity. Animals with overt thymic or generalized leukemias exhibited complete or pronounced depression of immune competence. The immune response of preleukemic mice was only moderately subnormal. It would seem that the immune competence of ≥9-month-old AKR mice is depressed because (1) the antigen-processing mechanism is defective, (2) the pool size of immunocompetent cells in the animals is reduced, or (3) the internal "milieu" or "environment" for support of differentiation and proliferation of antigen-stimulated immunocompetent cells is deficient.

Since in intact animals the full potential of immune competence may not be expressed because of overriding homeostatic mechanisms, the cell transfer system (spleen cells transferred to a lethally irradiated recipient), which allows maximum expression of immunocompetent cells, was used to resolve this problem. Studies carried out using both "primed" and "non-primed" spleen cells demonstrated that, when AKR/J or C3HeB/FeJ spleen cells were transferred with antigen (sheep red blood cells) to lethally irradiated young adult AKR/J recipients, the immune response in terms of peak numbers of direct and indirect PFC was low.

However, the immune response was high when the same number of the same spleen cells was transferred to lethally irradiated young adult C3HeB/FeJ recipients and intermediate when transferred to F_1 recipients.

These results suggest that the observed immunologic depression of nine-month-old AKR mice is not due to deficiency in the number of immunocompetent cells. Rather, they suggest that the internal environment of the AKR mouse for support of differentiation and proliferation of antigen-triggered immunocompetent cells is deficient. Deficiency in the antigen-processing mechanism seems unlikely, since comparable results were obtained with spleen hemopoietic colony-forming cells. The nature of this deficiency is under study.

Reference

[1] Pathology and Physiology section.

14.7 STUDIES ON MURINE PLASMA CELL TUMORS

J. F. Albright Toshihiko Sado

Certain strains of mice, when injected with irritating substances, develop plasma cell tumors. The tumors produce a unique gamma globulin, and this fact indicates that the tumor cells are clonally derived. In mice virtually all plasma tumor cells contain virus-like particles. It is assumed that there is a relationship between the virus and the origin and/or maintenance of the tumor cells; more specifically, the virus might be involved in directing synthesis of the specific globulin. We are investigating the properties of one plasma cell tumor, MOPC31c, which produces γF globulin.

The tumor is maintained by serial passage in BALB/c mice by either subcutaneous or intraperitoneal injection. Morphological examination of the solid tumor revealed several features. Two kinds of cells are present: a "light" cell that possesses a large nucleus, abundant mitochondria, a prominent Golgi apparatus, and, characteristically, an extensive "cap" around the Golgi of virus particles (Fig. 14.7.1*a,b*). Another type of cell, which has a characteristic of reticular cells and is more electron opaque after staining ("dark" cell), is also present. This latter cell also contains a "cap" of virus

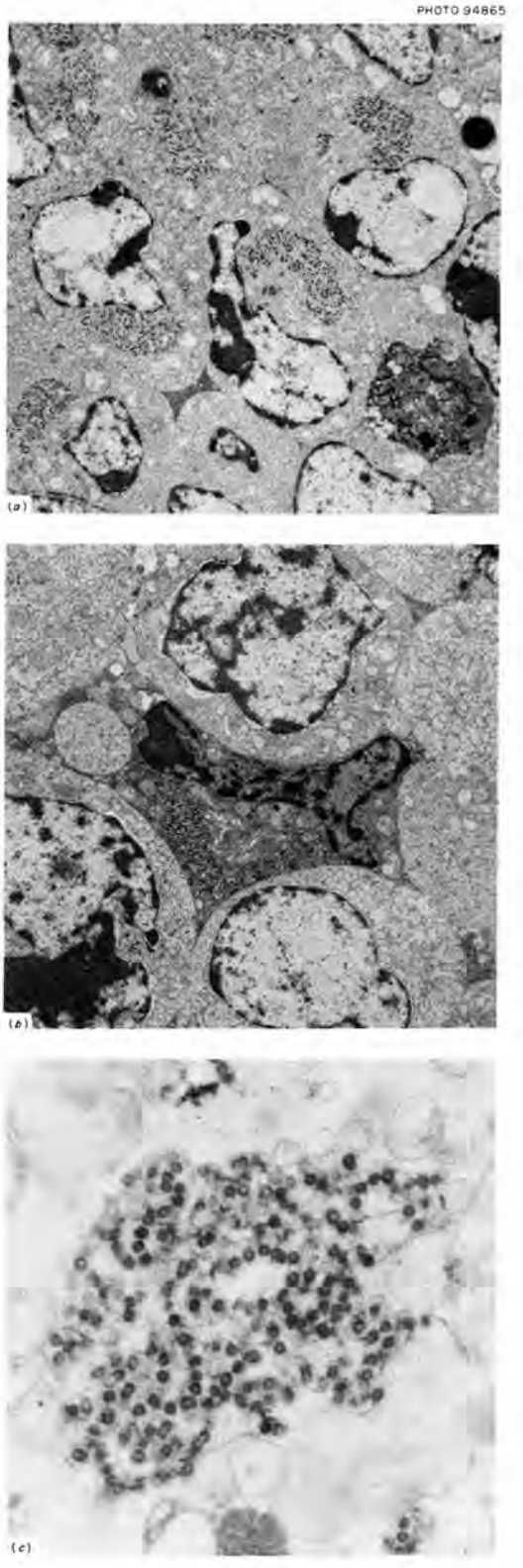

Fig. 14.7.1. Electron Micrographs of: (A) Section of Solid Plasma Cell Tumor Showing "Light" Cells That Feature Aggregates of Virus-Like Particles Arranged as a "Cap" Around the Golgi Apparatus (6728X); (B) Section Showing a "Dark" Cell with Accumulation of Virus-Like Particles Around the Golgi Apparatus (5590X); (C) Isolated Aggregate of Virus-Like Particles Enclosed in Sacs of Cellular Membranes, Presumably Smooth Endoplasmic Reticulum (38,930X). Reduced 47%.

particles around the Golgi. The viruses appear to be located in sacs of membranes within the cells. No extracellular virus particles were seen. Some information about the aggregates of virus particles and their association with the Golgi apparatus was obtained by homogenizing the tumor cells and studying the fractions obtained after centrifugation in a density gradient. The step gradient was composed of layers of a buffered sucrose-dextran mixture of different densities. Analysis by electron microscopy of the fractions revealed that (1) the virus aggregates and Golgi apparatus were distributed in different densities of sucrose and (2) the virus aggregates remained nearly intact and present in an extensive sac of membranes (see Fig. 14.7.1*c*). Enrichment of the virus aggregates resulted, although such fractions were contaminated by other cell particulate matter.

Some of the objectives of our current investigations are: (1) isolation of the virus, (2) determination of whether or not the virus is any of the common murine viruses and thus a "passenger" rather than a causative agent, (3) determination of whether or not the virus can induce the tumor, (4) determination of whether or not the virus is synthesized by the cells in which it is found, (5) establishment of new clones of the tumor in order to obtain virus-free cell clones and separate clones of the "light" and "dark" cells. Additional experiments are in progress aimed at the induction of the neoplasia in existing antibody-forming cells in order to obtain information about the type of cell in which the neoplasia arises.

14.8 GRADUAL LOSS OF IMMUNE COMPETENCE WITH OLD AGE

Takashi Makinodan W. J. Peterson

This program was initiated ten years ago. Based on a series of studies we concluded that in mice the immune system achieved a maximum in functional capacity during late juvenility and thereafter declined steadily with advancing age.[1] Various observations have implicated the failure of the immune system in senescence and death. Subsequently, several experiments have been initiated sequentially in an attempt to answer some of the many questions which arose as a consequence of our earlier findings. At this time the data of these ongoing studies are still incomplete and therefore will not be discussed.

During the past year another experiment was initiated, and it is concerned with the effect of insults on the immune system of young mice on their immune competence in later life and their life-span. We have been using x irradiation as the physical insult, cortisone and cytoxan (cyclophosphamide) as chemical insults, and splenectomy and thymectomy as surgical insults. It is known that the immune system of young mice exposed to most of these insults recovers rather rapidly, but there is no information on the long-term effects of these insults.

The reasons for undertaking this study are as follows. Hayflick[2] has shown that normal mammalian cells divide in vitro only a fixed number of times. Because the precursors of antibody-forming cells are proliferating cells, it was reasoned that these cells may also be programmed to proliferate only a fixed number of times. If so, the number of these precursor cells should begin to decrease earlier in adulthood among individuals whose precursor cells have been induced to undergo excessive numbers of division during their youth. Consequently, their immune system should decay earlier and possibly their mean life-span should be shortened.

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15. General Physiology

Radiation Biology	Molecular Biology
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15.1 INDUCTION OF CELL DIVISION IN BACTERIAL FILAMENTS BY CELL EXTRACTS

W. D. Fisher H. I. Adler[1] F. W. Shull, Jr.

Escherichia coli K-12 AB1899 NM forms long coencytic filaments after exposure to radiation. Such filaments grow and synthesize macromolecular constituents at a normal rate for several hours after irradiation but do not divide and eventually become inactive without forming macroscopic colonies. Treatment of growing populations with bacterial cell extracts stimulates cross septation in the filaments, and a certain fraction divide and produce macroscopically visible colonies.

The factor in the cell extracts which induces cell division is associated with a heat-labile particulate fraction which sediments at about 100 S and has a buoyant density in sucrose of 1.2 g/cm^3. Fractions purified by centrifugation contained 2% of the total protein of the extract but no measurable RNA or DNA. The extracts were inactivated by incubation with phospholipases, lipases, and detergents but not by incubation with selected proteases and nucleases. Frozen or lyophilized samples were stable to storage.

The enzymatic sensitivity and density of the division-promoting activity suggests that it may be membrane associated.

Reference

[1] Radiation Microbiology and Microbial Genetics section.

15.2 THE EFFECT OF CELL EXTRACTS ON THE GROWTH RATE OF BACTERIAL FILAMENTS

W. D. Fisher A. L. Thunberg

We have partially characterized a factor in bacterial cell extracts which can promote cell division in radiation-induced bacterial filaments.[1] Since it has been suggested that many agents which promote survival of bacterial cells after irradiation have in common the ability to inhibit growth and protein synthesis, we have examined the effect of cell extracts and selected chemicals on the growth rate of filaments of *E. coli* 1899 NM.

The rate of elongation of filaments of 1899 NM was measured microscopically from camera lucida tracing of cells plated on nutrient agar slides. The rate of elongation of filaments plated on agar containing an amount of cell extract which causes maximum stimulation of cell division was identical with the growth rate of control filaments. However, chemical agents such as pantoyl lactone, which can also induce cell division, markedly inhibited growth. Therefore the effect of the extracts, unlike certain chemical agents, is not associated with a measurable growth delay.

Reference

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15.3 POLYURIDYLIC ACID-DIRECTED PHENYLALANINE INCORPORATION IN MINICELL EXTRACTS

J. A. Fralick[1] W. D. Fisher H. I. Adler[1]

Cells of *Escherichia coli* K-12, P678, frequently undergo an aberrant cell division by forming cross walls near the ends of the cell. These misplaced cell walls result in the production of small, spherical, anuclear structures called "minicells," which are deficient in DNA. DNA can be introduced into minicells by conjugation with *E. coli* donor strains. To date, we have been unable to demonstrate in vivo protein synthesis in either conjugated or nonconjugated minicells. As reported elsewhere,[2] minicells have also been shown to be deficient in DNA-dependent RNA polymerase, and the lack of protein synthesis can be explained on this basis. We have therefore investigated the ability of synthetic message to promote ^{14}C-phenylalanine incorporation in minicell extracts to determine if the remainder of the protein-synthesizing system in minicells is intact. Our results show that the minicell extract is capable of utilizing polyuridylic acid to incorporate phenylalanine at a level comparable with normal *E. coli* cell extracts, suggesting that minicells could carry on in vivo protein synthesis if provided with a messenger RNA or messenger-RNA-generating system.

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16. Cell Physiology

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S. P. Leibo	Carol A. Opple[c]
J. F. Brandts[a]	Guy Johnson III[d]
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[a]Consultant
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[d]Temporary

16.1 NUCLEATION OF SUPERCOOLED WATER BY MACROMOLECULES IN SOLUTION

Peter Mazur R. H. Miller

Several investigators have suggested that the hydration layer around some proteins and nucleic acids may be ordered or "icelike."[1,2] If it were truly icelike, it might cause supercooled water to freeze at higher temperatures than it does spontaneously. The present study is concerned with testing that supposition. By the use of Millipore filters, a laminar flow hood, and scrupulous rinsing of all glassware with filtered water, it has been possible to consistently supercool 0.02-ml samples of water or dilute salt solutions to −20 to −21°C. The experiments involved a comparison of the supercooling temperature of 0.02-ml samples of dilute solutions of DNA and collagen (usually 10 μg/ml) in dilute NaCl (0.007 *M*) or citrate buffer (0.003 *M*) with the supercooling temperature of the salt solutions alone.

The DNA exerted no effect. The mean extent of supercooling was 20.5, 21.0, and 21.4°C for native DNA (calf thymus), denatured DNA (10 min at 90°C), and 0.007 *M* NaCl respectively. However, collagen (calf skin) appears to exert a large effect. The mean supercooling in the presence of the macromolecule was only 14.1°C vs 20.5°C for the citrate buffer control. We have not yet proven that it is the collagen, and not some impurity, that is responsible for the higher nucleating temperature, but the ability of the solutions to nucleate disappeared when they were heated above 50°C for 10 min.

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[2] H. J. C. Berendesen and C. Migchelsen, *Federation Proc.* **25**, 998 (1966).

16.2 FREEZING AND THAWING OF CHINESE HAMSTER CELLS: INTERACTIONS BETWEEN THE OPTIMUM COOLING VELOCITY FOR SURVIVAL AND THE NATURE AND CONCENTRATION OF PROTECTIVE ADDITIVE

Peter Mazur S. P. Leibo
John Farrant E. H. Y. Chu[1]

Current cryobiological practice[2] assumes that to obtain significant survival, mammalian cells must be suspended in solutions containing a low-molecular-weight permeating additive and frozen at about 1°C/min. Current theory states that the action of the additive is essentially colligative. It reduces the extent to which intra- and extracellular electrolytes concentrate during freezing, and it reduces cell dehydration.

However, recent results with Chinese hamster tissue culture cells are not consistent with this hypothesis. Furthermore, the usual freezing procedures produced poor survivals. The cells were suspended in Hanks' balanced salt solution or in Hanks' plus various concentrations of glycerol, sucrose, or PVP. Samples were frozen at −196°C at controlled rates varying from 1.5 to 585°C/min and generally thawed rapidly at 1100°C/min. The optimum cooling rate was between 20 and 350°C/min, depending on the concentration and type of additive. Of all the rates studied, a cooling velocity of 1.5°C/min was the most deleterious. The best survivals of slowly cooled hamster cells were obtained when the cells were suspended in 0.35 or 0.70 *M* sucrose or in 15% PVP. This was so in spite of the fact that cell volume measurements showed that sucrose cannot permeate the cells and the fact that the PVP probably had too high a molecular weight (40,000) to permeate. Protection thus not only did not require permeating of the additive, but it also cannot be ascribed solely to colligative mechanisms. Thus survival was much better with 3×10^{-4} *M* PVP (15% w/v concentration) than with 1.25 *M* glycerol (12% w/v concentration).

The survival of cells cooled slower than 11°C/min in 15% PVP was unaffected by the thawing rate, but that of more rapidly cooled cells was much lower when subsequent warming was moderately slow (30°C/min) than when it was rapid (1100°C/min). This finding suggests that intracellular ice did in fact form in rapidly cooled cells but that this ice became injurious only when slow warming permitted recrystallization to occur.

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16.3 FREEZING OF BACTERIOPHAGE T4: TEMPERATURE AND RATE EFFECTS AS A FUNCTION OF SALT CONCENTRATION

S. P. Leibo Peter Mazur

When frozen in dilute NaCl solutions, phages T4B and T4D are much more sensitive to rapid thawing than their corresponding osmotic-shock-resistant mutants T4B*os* and T4D*os*. We have hypothesized that the inactivation of shock-sensitive phage when rapidly warmed is due to the osmotic shock that occurs during rapid thawing, as salts concentrated during freezing are rapidly diluted.[1] This hypothesis has now been strengthened by a genetic analysis demonstrating that a mutant isolated by freezing and rapid thawing (*op* mutant) is identical to a shock-resistant mutant isolated by osmotic shock (*os* mutant). This analysis was based on the demonstration that mixed infections under restrictive conditions with an *os* mutant and either a *ts* phage (mutant in gene 24) or either of two *am* phages (both mutant in gene 24) yield only phages that are phenotypically *os*. These experiments confirmed the preliminary conclusion[2] that the *os* mutation is located in gene 24. Similar complementation tests have demonstrated that the *op* mutation is also located in gene 24 and is probably identical to the *os* mutation.

However, in contrast to the situation in dilute solution (<0.1 *m*), phages T4B and T4B*os* were equally sensitive when frozen in concentrated NaCl solutions regardless of cooling or warming rates. Phage inactivation under these conditions was a step function of subzero temperature, just as is the complete crystallization of water and the precipitation of all salts in a solution undergoing freezing. Therefore the complete solidification of the phage suspension apparently is a major factor in the inactivation of T4 phage frozen in solutions 0.1 *m* or greater in concentration.

References

[1] S. P. Leibo and Peter Mazur, *Virology* (submitted for publication).

[2] S. P. Leibo *et al., Biol. Div. Ann. Progr. Rept. Dec. 31, 1967,* ORNL-4240, p. 200.

16.4 FREEZING OF MOUSE MARROW STEM CELLS: INTERACTIONS OF COOLING AND WARMING RATES IN THE PRESENCE OF PVP, SUCROSE, OR GLYCEROL

S. P. Leibo Peter Mazur
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L. H. Smith[2]

Based on results with micro-organisms, one theory of freezing damage in cells states that the survival of a frozen-thawed cell is critically dependent on the rate at which it is cooled to subzero temperatures.[3] The hypothesis suggests that two factors interact, as functions of cooling rate, to determine the ultimate survival of a frozen and thawed cell: (1) exposure to solution effects such as increased electrolyte concentration, pH changes, and dehydration; and (2) the formation of intracellular ice during cooling, and the extent of its

recrystallization during warming. Using the colony-forming ability of the stem cells of mouse marrow as an assay of viability, we have sought to test the applicability of this theory to freezing damage in a nucleated mammalian cell.

Stem cells suspended in each of three concentrations of glycerol, two of polyvinylpyrrolidone (molecular weight = 40,000), or one of sucrose were frozen at rates varying from 0.3 to 500°C/min to −196°C, held for about 1 hr, and then thawed rapidly at about 900°C/min. In one experiment, cells cooled either rapidly or slowly were warmed either rapidly or slowly. Under all conditions the survival of the colony-forming ability of the stem cells varied as a function of cooling rate, showing a distinct maximum at one rate. But the optimum rate varied with the specific additive and its concentration. For example, maximum survival was obtained for cells cooled at 1.5°C/min in 1.25 *M* glycerol, at 15°C/min in 0.8 *M* glycerol, and at 100°C/min in 0.4 *M* glycerol. If cells in 1.25 *M* glycerol were cooled at 1.5°C/min, survival was 50% regardless of whether the suspensions were thawed at 1°C/min or at 500°C/min; but if these cells were cooled at 300°C/min, survival was 30% when the cells were thawed at 500°C/min and only 2% when they were thawed at 1°C/min. Good survival was also obtained in two additives that we believe to be nonpermeating, namely, PVP and sucrose. Here, too, survivals showed distinct maxima as a function of cooling rate. Moreover, since the maximum survival was 45% for cells suspended in 0.35 *M* sucrose but only 20% for cells in 0.4 *M* glycerol, it appears that a nonpermeating additive is more effective on a molar basis in preventing freezing damage than is glycerol, which has been assumed to permeate nucleated cells.

These results are consistent with the theory of freezing damage developed from findings with microorganisms; that is, cells cooled at rates slower than that giving maximum survival are damaged by solution effects, and cells cooled at rates faster than that giving maximum survival are damaged by intracellular ice formation. The extent of damage to cells containing intracellular ice will depend on the extent of recrystallization, a phenomenon known to increase with decreasing warming rate.

References

[1] AEC-NCI Cocarcinogenesis Program.

[2] Mammalian Recovery section.

[3] Peter Mazur, *Federation Proc.* 24, S-175 (1965).

17. Chemical Protection and Enzyme Catalysis

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[a]Dual Assignments
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17.1 EFFECT OF SMALL DOSES OF SYNGENEIC BONE MARROW ON THE 30- AND 90-DAY LD_{50} OF X-IRRADIATED MICE

D. G. Doherty L. H. Smith[1]
Frances E. Hacker

Introduction. – There are numerous data showing that 30-day survival of lethally irradiated mice is a function of the number of marrow cells injected. Doherty[2] determined the $LD_{50/30}$ and $LD_{50/90}$ as a function of log marrow cell dose and found that the increase in LD_{50} had two linear phases. The first of the two phases had a larger slope and occurred over a cell dose range of 0.05 to 0.2 X 10^6, wherein the $LD_{50/30}$ increased from 853 to 1220 r. The second phase occurred over a cell dose range of 0.2 to 200.0 X 10^6, wherein the $LD_{50/30}$ increased from 1220 to 1450 r. The $LD_{50/90}$ values were 2 to 5% lower. Thus bone marrow transplantation in mice is most efficient, in terms of promoting recovery, at exposures below 1200 r, relatively less being gained by even large cell doses when exposures are in excess of 1200 r. To complement these data we studied the effects of very small marrow cell doses on 30- and 90-day LD_{50}, principally in the LD_{10} to LD_{100} x-ray range.

Materials and Methods. – Female (C57BL/Cum ♀ X C3H/Anf ♂)F_1 mice were used. X-ray exposures ranged from 700 to 1000 r for cell doses of 0.0005 to 0.025 X 10^6 and from 750 to 1300 r for 0.075 X 10^6 cells. The minimum number of mice per cell-dose point was 50, although frequently 60 to 90 were used. Control points in the 650-to-1000-r range represent on the average 200 mice each, and in the 950-to-1300-r range, 60 mice each.

Results and Discussion. – From the probit analysis of survival data, the LD_{50} were obtained. Plotting $LD_{50/30}$ as a function of log cell dose generates the curve of Fig. 17.1.1, which also includes data (dashed line) from previous experiments.[2] Where data from the two series of experiments overlap (0.025 to 0.1 X 10^6 cells), there is a good correspondence for slopes but only fair correspondence for absolute values. Considering data from both series, there are three phases which relate $LD_{50/30}$ to cell dose. Phase 1 occurs over the range of 0.0005 to ~0.030 X 10^6 cells and has the shallowest slope. Phase 2 occurs over the range of ~0.030 to 0.2 X 10^6 cells and has the steepest slope. The third phase occurs over the range of 0.2 X 10^6 to at least 200 X 10^6 cells and is intermediate in slope. From these data we conclude that in terms of 30-day survival, syngeneic marrow is most efficient over a relatively narrow dose range, that is, slightly less than one decade of cell dose represented by phase 2. The break between phases 2 and 3 probably reflects an increasing number of deaths resulting from intestinal damage, which becomes more critical as the x-ray exposure increases. The reason for the shallowness of phase 1 and for the apparent break between phases 1 and 2 is not clear, but may be related to insufficient hemopoiesis generated by small marrow doses. A curve for the $LD_{50/90}$ data is not presented because for the

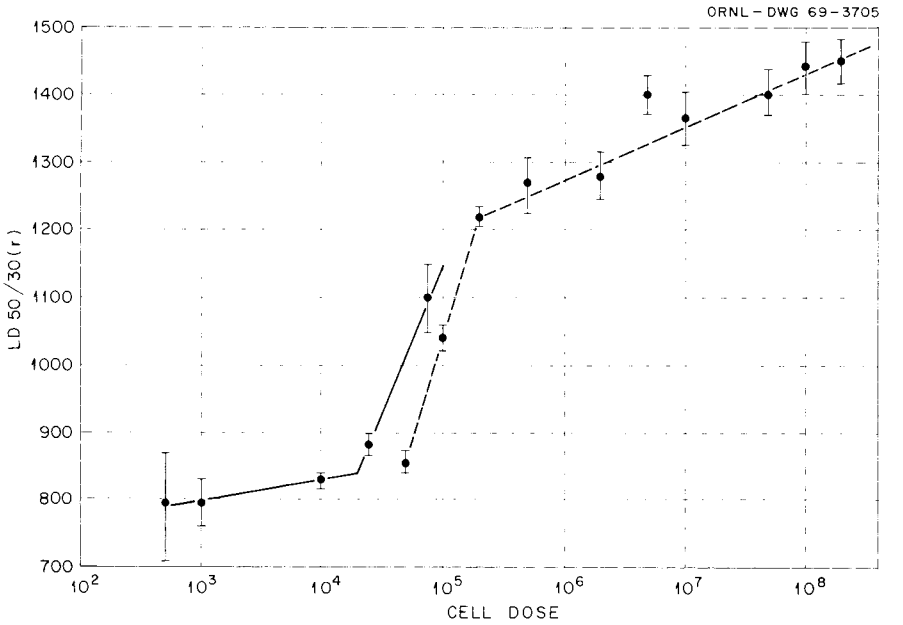

Fig. 17.1.1. LD 50/30 as a Function of Syngeneic Bone Marrow Cell Dose. Solid line represents present data; dashed line represents data from Doherty.[2] Vertical bars are 95% confidence limits.

present experiments 30- and 90-day survival values are essentially the same except for mice exposed to 1100 r or more. In these groups (1100, 1150, and 1200 r) 90-day survival percentages were about one-half of those scored at 30 days. Late deaths at higher exposures are reflected in the fact that for the 0.075×10^6 cell dose group, the $LD_{50/90}$ is considerably lower than the $LD_{50/30}$.

Results of the present experiments together with previous data[2] indicate a triphasic response of the $LD_{50/30}$ to the dose of syngeneic marrow cells, which seem to be most efficient over somewhat less than a decade of cell dose.

In addition to the fact that marrow injection increases the LD_{50}, the slope of the survival curve for marrow-injected mice is about half that for x-ray controls. This was evident at all marrow doses tested. The slope, however, is not a function of cell dose, although apparently there is a threshold because slopes for cell doses of 0.025×10^6 cells or less were not different from that of x-ray controls. It is not clear why the slopes of survival curves for marrow-injected mice are less than that for x-ray control mice.

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[1] Mammalian Recovery section.

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17.2 STUDIES ON THE SUBSTRATE SPECIFICITY OF PEPSIN

D. G. Doherty Jesse James
Margaret A. Turner

The nature of the active center of pepsin continues to be a matter of considerable controversy and challenge. Using kinetic techniques we have previously established the stereochemical requirements as well as the importance of the *N*-acyl group of dipeptides in determining the binding and susceptibility to pepsin action. In the realm of uncharged *N*-acyl groups the chloroacetyl group was found to be an excellent blocking group for the *N*-terminal. The hydrolysis rates for chloroacetyl-L-Phe-L-PheOEt and free acid were considerably faster than the acetyl compounds. Bromoacetyl and iodoacetyl groups were equally effective. The kinetic constants for chloroacetyl-L-Phe-L-PheOEt were K_m = 0.056 mM, k_3 0.045 sec^{-1}, and k_3/K_m 0.68 sec^{-1} mM^{-1}. Using this group the nature of the side-chain binding was explored by preparing the following new series of dipeptide esters: -L-Leu-L-PheOEt, -L-Phe-L-LeuOEt, L-Leu-L-LeuOEt, -Gly-L--PheOEt, L-Ala-L-PheOEt, -L-Ile-L-PheOEt, -L-Nle-L-PheOEt, L-Val-L-PheOEt, and L-Met-L-PheOEt. The most susceptible peptide of this group was -L-Leu-L-PheOEt, with kinetic constants of K_m 0.50 mM, k_3 0.034 sec^{-1}, and k_3/K_m 0.066 sec^{-1} mM^{-1}.

The corresponding acetyl dipeptide was not hydrolyzed, nor was chloroacetyl-L-Phe-L-LeuOEt. The only other peptide cleaved at a measurable rate was chloroacetyl-L-Nle-L-PheOEt. These findings indicate that the *N*-terminal amino acid residue must be hydrophobic but not necessarily aromatic in nature, while there is an obligatory requirement for an aromatic residue at the *C*-terminal site. In an attempt to find a peptide with a partially fixed likely conformation we synthesized D- and L-3-carboxydihydroisostyril-L-PheOEt. Neither of these compounds was cleaved. The conformational aspects of the haloacetyl dipeptides are being explored by x-ray crystallography in collaboration with C. H. Wei of the X-Ray Diffraction group.

18. Pathology and Physiology

Section Chief – A. C. Upton[a]

Pathological Effects of Neutrons, High-Energy Radiation, and Chemicals (18.1–18.5)

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N. K. Clapp[a,b]
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Hematology and Radiation Physiology (18.8–18.10)

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Gnotobiotics (18.11, 18.12)

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Virology (18.13, 18.14)

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Clinical Biochemistry and Microbiology (18.15)

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Histology and Autoradiography (18.16)		*Participation in Cooperative Programs:* AEC-NCI Cocarcinogenesis (30.9–30.12, 30.31, 30.32)
W. D. Gude	Sandra Bradshaw Joyce G. Feezell Mary S. Gude Tennyson Mack Dorothea L. Parker Helen Seal J. W. Wesley Patricia Wortham[a] Violet D. Wright Anne H. Jones[d]	

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18.1 LOW (MEDIUM) LEVEL EXPERIMENT

All Members of Pathology-Physiology Section

Introduction. – The primary objective of this experiment is to determine the dose-response relation for radiation-induced carcinogenesis and life shortening under conditions of low-level irradiation in the mouse. It is also the aim of the experiment to obtain correlative data on the biochemical, physiological, microbiological, and morphological changes associated with late somatic effects of radiation and aging.

Previous progress reports have described the protocol of the experiment, the controlled environment in which experimental mice will be maintained, methods for detecting changes in the selected indigenous bacteria of the mouse intestine, the continuous absence of conventional mouse viruses, preliminary results with respect to longevity and incidence of leukemia, and a method for electromechanically recording and retrieving breeding data and preexperimental information for each mouse.

Results and Discussion. – More than 11,000 experimental mice have been set up thus far (Table 18.1.1). The first such animals were set up in 1964 as part of a pilot experiment (stage I) to verify that the facilities, methods, and mice were suitable for the purpose in question and to permit refinement of methods and training of personnel as required. All animals in this stage of the experiment are now dead, and the corresponding results give no indication that the barrier-sustained mice developed for the study will be unsatisfactory for the purposes in question. They do, however, indicate that mice of the barrier-sustained subline have a higher incidence of radiation-induced lymphomas and a lower incidence of granulocytic leukemias than do their conventional counterparts. The lower incidence of myeloid leukemia in the barrier mice, which is consistent with results in germfree mice and mice of another barrier-sustained colony, may tentatively be attributed, at least in part, to the pathogen-free nature of the microflora in the barrier-sustained mice. The lower incidence of granulocytic leukemia is paralleled by a higher incidence of lymphoid leukemia, with the result that the combined incidence of both neoplasms in the new subline is similar to that in the old. The barrier mice also differ from their conventional counterparts in faster rate of growth and longer life-span; that is, the mean survival time of nonirradiated barrier mice was 668 days in males and 603 days in females, as compared with 572 days and 581 days in nonirradiated conventional males and females.

Preceding completion of the first stage and preparations for the main experiment, more than 1300 male and female mice were set up during 1966–67 in a second stage of the experiment (Table 18.1.1). Over 75% of these mice have now died, and the preliminary results they have yielded agree closely with the results of the first stage of the experiment.

Because the results of these preliminary stages were sufficiently definitive by August 1967 to verify that the protocol, procedures, and facilities for the main experiment were satisfactory, irradiation of mice for this phase of the study (stage III) was begun at that time

Table 18.1.1. Irradiation and Mortality to Date in the Successive Stages of the Low (Medium) Level Experiment

Dose (rads)	Number of Mice Dead/Number Set Up					
	Stage I		Stage II		Stage III	
	Males	Females	Males	Females	Males	Females[a]
0	74/74	46/46	54/89	100/134	50/337	192/2186[b]
10	0	0	0	0	36/255	157/1660
25	0	0	0	0	19/96	61/539
50	43/43	58/58	101/160	169/196	15/86	81/542
100	48/48	36/36	97/139	140/166	16/84	94/541
150	77/77	80/80	83/123	102/110	24/85	120/539
300	73/73	67/67	60/70	128/130	109/331	770/2212[b]
Total	315/315[c]	287/287[c]	395/581	639/736	269/1274	1478/8594

[a]Numbers set up thus far represent approximately 55% of the total numbers called for at each dose level.

[b]Number includes mice for serial sacrifice (only ~500 mice will be killed, but ~4000 need to be set up to provide survivors for sacrifice at intervals up to age of 30 months; the remaining ~3500 will die naturally and are thus comparable with the mice of all other dose groups).

[c]Comparable numbers of conventional mice irradiated at corresponding dose levels are not included in this table.

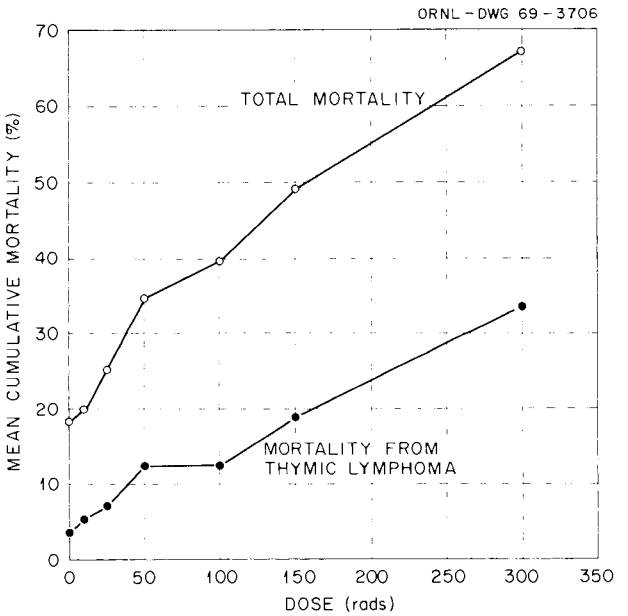

Fig. 18.1.1. Preliminary Data on Overall Mortality and Mortality from Thymic Lymphoma in Female Mice of the Main Stage (III) of the Experiment Irradiated to Date, as Analyzed 14 Months After Irradiation.

and has continued since then. To date, slightly more than one-half of the animals called for in this phase of the experiment have been set up in weekly replicates. Of these, a considerable number have already died or been sacrificed (Table 18.1.1). Although data on pathologic effects in these animals are highly preliminary, they disclose an increase in mortality with dose, as expected (Fig. 18.1.1). Of considerable interest is the evidence suggesting the existence of a small effect even at the lowest dose tested, namely, 10 rads. Such evidence is, however, highly preliminary and will require substantially larger numbers of animals for verification.

18.2 PRELIMINARY DATA ON THE RELATIVE BIOLOGICAL EFFECTIVENESS OF 60-Mev PROTONS FOR LEUKEMIA AND TUMOR INDUCTION IN FEMALE RF MICE[1]

E. B. Darden, Jr. N. K. Clapp
A. C. Upton M. C. Jernigan

Introduction. — To study the relative biological effectiveness of 60-Mev protons for induction of neoplasms and other late somatic effects, about 1600 eight-week-old RF female mice were subjected in replicates to a single whole-body dose of 0, 50, 100, 200, 300, or 400 rads of 60-Mev protons in the Oak Ridge Isochronous Cyclotron. Similar numbers of mice of the same age, sex, and strain were exposed to comparable doses of 300-kvp x rays. Following irradiation the mouse cages were inspected one or more times daily for dead and moribund animals. A complete necropsy, including cranial contents, has been performed on more than 98% of the mice that have died to

date, and in two-thirds or more of the cases tissues have been obtained for histological examination.

Results and Discussion. – With less than 1% of the 30-day survivors still living, the gross findings suggest that dose-incidence relations for thymic lymphoma, myeloid leukemia, other late-occurring leukemias, pulmonary adenomas, and ovarian tumors are similar in the proton- and the x-irradiated mice. The situation appears to be comparable for other neoplasms, but the latter have been observed only sporadically. These preliminary data on oncogenic effects, although subject to minor changes pending histological verification, imply an RBE of approximately 1 for 60-Mev protons and are thus consistent with our previously reported findings[2,3] on life shortening, lens opacification, weight gain, and acute effects ($LD_{50/30}$, mean survival time, and organ weights) in mice of the same strain.

References

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[3] J. L. Montour *et al., Biol. Div. Ann. Progr. Rept. Dec. 31, 1967,* ORNL-4240, pp. 363–64.

18.3 INFLUENCE OF ALTITUDE ON LATE EFFECTS OF RADIATION IN RF/UN MICE: OBSERVATIONS ON SURVIVAL TIME, BLOOD CHANGES, BODY WEIGHT, AND INCIDENCE OF NEOPLASMS

Pablo Mori-Chavez[1] A. C. Upton
Maximo Salazar J.[1] J. W. Conklin

Introduction. – Since hypoxia during irradiation, which decreases the radiosensitivity of cells and tissues, protects to some degree against radiation-induced life shortening, influences the development of spontaneous, estrogen-induced, and urethan-induced neoplasms, enhances the growth of some transplantable tumors, and increases the frequency of their metastases, this investigation was undertaken to study the effects of hypoxia induced by high altitude after irradiation on the development of neoplasms and other late somatic effects of radiation in mice.

Results and Discussion. – RF female mice were subjected to sham irradiation (as the control), or to 150 or 300 r of whole-body x rays at ten weeks of age and subsequently kept for the duration of life either at sea level or at high altitude (14,900 ft). On ascent to high altitude the mice showed a rapid, marked erythropoietic response, which was not detectably impaired by earlier irradiation. There was a further gradual increase in the erythrocyte count and hemoglobin level with advancing age at high altitude, whereas the reverse occurred at sea level. With advancing age the granulocyte count also increased while the mononuclear cell count decreased, neither change being detectably affected by irradiation or altitude. The life-span decreased with increasing radiation dose and was shorter in all groups (including controls) at high altitude than at sea level. Mice at high altitude also showed impairment in body-weight gain and a lower overall incidence of neoplasms than did the mice at sea level. The incidence of thymic lymphomas and granulocytic leukemias increased with irradiation, whereas the reverse was true of other lymphomas and leukemias. In general, at any given dose level, all lymphomas and leukemias were less common at high altitude than at sea level. The incidence of lung tumors decreased with irradiation at sea level but increased at high altitude, especially when the incidence was adjusted to correct for differences in survival time. Pulmonary carcinomas were many times more common at high altitude than at sea level. The incidence of ovarian tumors was increased by irradiation but was unaffected by altitude. Ovarian telangiectatic and angiomatoid lesions, however, were more prevalent at high altitude than at sea level. Miscellaneous neoplasms of other types and sites were observed sporadically in all experimental groups, but at a frequency too low to disclose any definite effects of irradiation and altitude.

Reference

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18.4 IMPAIRMENT OF HEMOPOIETIC SPLEEN COLONY FORMATION IN AKR MICE

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Takashi Makinodan[1] Lou C. Satterfield

Introduction. – In exploring the impairment of immunological responsiveness noted in otherwise normal AKR mice, we have analyzed the ability of such mice to form hemopoietic spleen colonies as compared with mice of other strains. For this purpose, marrow and spleen cells have been exchanged between 11-

week-old female mice of the AKR/J, C3HeB/FeJ, and (C3HeB/FeJ × AKR/J)F1 strains, with all donor-recipient combinations of these three strains being tested. Recipients were exposed to 900 r, promptly injected iv with 10^5 to 10^7 marrow or spleen cells, and sacrificed six days later for microscopic analysis of the resulting hemopoietic spleen colonies.

Results and Discussion. – There was little or no variation in colony-forming ability among donors of the different strains but significant variations among recipients; that is, irrespective of the strain of the donor, fewer and smaller colonies were found in AKR recipients than in C3H recipients, with intermediate results in F1 recipients.

These observations are consistent with observed differences in immunological reactivity among mice of the same strains, implicating a deficiency in the internal environment of the AKR mouse rather than a deficiency in the responsiveness of its stem cells, given a favorable environment. The nature of the deficiency, its mode of transmission, the role of genetic and viral factors in its pathogenesis, and its possible relation to leukemogenesis in the leukemia-prone AKR mouse are under study.

Reference

[1] Radiation Immunology section.

18.5 EFFECTS OF MICROBIAL ENVIRONMENT AND ENDOTOXIN ON HEMOPOIESIS IN SUBLETHALLY IRRADIATED RFM MICE

V. K. Jenkins H. E. Walburg, Jr.
A. C. Upton Lou C. Satterfield

Introduction. – Previous studies in this laboratory have revealed that susceptibility to radiation-induced granulocytic leukemia is reduced in germfree and barrier-derived RFM mice, as compared with their conventional counterparts. The effects of the microbial environment and of bacterial endotoxin on regeneration of granulocytopoiesis after whole-body irradiation in mice were studied to explore further the basis of the observed differences in susceptibility to radiation leukemogenesis.

Results and Discussion. – Young adult male and female RFM mice housed under germfree, barrier-derived, and conventional conditions were exposed to 350–500 rads of acute whole-body x radiation and sacrificed six days later for microscopic analysis of hemopoietic spleen colony formation. The proportion of granulocytopoietic colonies was higher, and the proportion of erythropoietic colonies correspondingly lower, in (1) germfree mice and barrier-derived mice than in their conventional counterparts and (2) barrier-derived mice injected with endotoxin one day before irradiation than in noninjected controls. In addition to affecting the relative numbers of different colonies, endotoxin also increased the absolute numbers of colonies of all types. The total number of colonies was likewise slightly higher in conventional than in germfree mice.

These results indicate that bacterial endotoxin and the microbial environment are both capable of influencing erythropoiesis and granulocytopoiesis differentially, as well as influencing overall hemopoietic repopulation, in sublethally irradiated mice. In addition, these results are consistent with the hypothesis that susceptibility to induction of granulocytic leukemia is correlated with the number and turnover of granulocytopoietic cells in the irradiated animal.

18.6 RABBIT SYNCYTIUM VIRUS: ULTRASTRUCTURAL STUDIES

R. C. Brown C. W. Shaw[1]
J. A. Morris[1] M. D. Bloomer

Introduction. – The preparation of virus vaccine for human use should include efforts to detect and eliminate, or inactivate, all viruses potentially pathogenic for man. Since it is proposed that rubella vaccine be produced in rabbit kidney cell cultures,[2] it is of importance to learn as much as possible about viruses indigenous to this animal that might contaminate such cultures. The inclusion of only six viruses of rabbit origin in current lists of animal viruses indicates how very little is known about viruses of this animal. Virtually nothing is known of their potential as agents of disease for man. Recently we[3] described a previously unrecognized virus – rabbit syncytium virus – which was isolated from an apparently healthy wild cottontail rabbit. On the basis of chemical, physical, and biological properties, we suggested that this new virus should be tentatively included in the mumps–Newcastle disease–parainfluenza–respiratory–syncytial virus complex.

In order to understand better the nature of rabbit syncytium virus and to classify it more precisely, we examined its ultrastructure, mode of replication, and cellular interactions in infected baby hamster kidney (BHK-21) and primary canine kidney cell cultures.

Results and Discussion. – Twenty-four hours after virus inoculation, cells in culture showed virus-related

cytopathic changes, beginning with cytoplasmic vesiculation and nuclear chromatin margination and progressing to kill almost all the cells after three to six days.

Two cellular structures related to virus infection were detected. One consisted of a rounded 650-A-diam non-membrane-bound particle structured from outside inward as follows: (1) ill-defined, moderately electron-dense 50- to 100-A-thick coat, (2) 50-A-thick unit-like membrane, and (3) a center consisting of a very electron-dense 200-A-diam central part and a moderately electron-dense 100-A-thick peripheral part. Negative stain preparations showed a "nodular" or "spiked" periphery suggestive of capsomeres. The second structure was a unit-membrane-bound rod of 750 to 1100 A diameter and of indeterminant length. The rods contained 100- to 150-A-diam, peripherally located ribosome-like structures in a relatively electron-lucent finely granular substance. The latter structures have not yet been identified in negative stain preparations.

The spherical particle originated in commonly found dense, granular cytoplasmic inclusions; rarely it was also found singly and in small clusters in the nuclei and in membrane-bound cytoplasmic vesicles into which it was seen to pass from the cytoplasm, acquiring a coat of the vesicle membrane in the process. The rod forms were often found in haphazardly arranged dense masses just beneath the cell membrane. Rods were less often singly and in groups within nuclei. They were not identified extracellularly and were not seen to pass into or out of cells or to bud from cell membranes. Often the "indefinite end" of a rod was closely associated with dense, granular material in which the spherical particle was being produced.

The BHK-21 cell line contained an indigenous rounded 1000- to 1100-A-diam virus-like particle which was seen to bud into cytoplasmic vesicles. This particle, which has been detected in this cell line by others, had a characteristic 250- to 300-A-thick, medium-electron-dense segmented periphery overlying a unit-like membrane which confined an electron-dense nucleoid.

In its structure, intracellular distribution, and apparent mode of replication, the rabbit syncytium virus is unlike hitherto described viruses, with the exception of the virus of epizootic diarrhea of infant mice (EDIM).[4] In contrast to EDIM, however, the spherical form of rabbit syncytium virus occurs in cell nuclei, and the rod structure contains ribosome-like particles, is larger than its spherical form, is absent from cytoplasmic vesicles or extracellular locations, and is present in dense cytoplasmic accumulations. In addition, certain cultural features of EDIM distinguish it from rabbit syncytium virus.

Based therefore on our determination of its morphology, intracellular distribution, and apparent mode of replication, as well as on previously determined chemical, physical, and biological properties, the rabbit syncytium virus does not fit into any of the 11 classifications of animal viruses. Although rabbit syncytium virus has not been shown to be pathogenic for rabbits,[3] however, its possible pathogenicity for man remains undetermined. Therefore it is important to detect and eliminate or inactivate it in cell cultures used for production of vaccine destined for use in man.

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18.7 MALACOPLAKIA OF THE TESTIS: ELECTRON MICROSCOPY AND ELECTRON DIFFRACTION STUDIES

R. C. Brown

Introduction. – Malacoplakia is a rare disease of obscure etiology, the occurrence of which outside the urinary tract has only recently been recognized. In our study of the histopathology of 8 new cases of malacoplakia of the testis,[1] found in a review of over 200 cases of granulomatous orchitis in the files of the Armed Forces Institute of Pathology, we were struck by the association of thrombosis and gram-negative bacteria with the lesions. The lesion in the testis, like that in the urinary tract, is a chronic granulomatous inflammation, with characteristic intracellular, concentrically laminated, 3- to 5-μ-diam mineral spherules staining positively for iron and calcium. In an effort to elucidate the structure, site of intracellular origin, and composition of the mineral spherules and to examine the ultrastructure of testicular lesions, we performed electron microscopic and electron diffraction studies on lesions selected from the best preserved tissues from our previously reported cases of malacoplakia of the testis.

Results and Discussion. – Despite the fact that our preserved tissue was not properly fixed for electron microscopy and had been in preservative for long periods (some up to ten years in 10% formalin), we were able to obtain considerable information by ultrastructural examination. The mineral spherules (Michaelis-Gutmann bodies) appeared to originate in rounded,

membrane-bound intracytoplasmic structures morphologically consistent with lysosomes. These appear to begin as one or more small clusters of slender, pointed, needle-like elements and to progress to larger, rounded, concentrically laminated, electron-dense and relatively electron-lucent bodies. Selected-area electron diffraction studies of thin sections of Epon-embedded material showed a diffraction pattern suggesting that the main diffracting component of the spherules is apatite. The spherules were confined to the large "histiocytic" cells with PAS-positive cytoplasmic granules. These large "histiocytic" cells also contained numerous myelin figures within cytoplasmic vesicles. The plasma cells, fibroblasts, and lymphocytes of the lesions were not different from normal. We did not recognize viruses or bacteria in the examined tissues, but our failure to confirm the presence of bacteria by electron microscopy was not unexpected, due to the limited sampling and the focal distribution of bacteria shown by light microscopy.

Although the etiology of this inflammatory disease remains obscure, the association of bacterial infection with an altered calcium-phosphorus metabolism might explain both the inflammation and the pathologic mineralization which are characteristic features of the disorder.

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18.8 MEGAKARYOCYTOPOIESIS IN MICE

T. T. Odell, Jr. C. W. Jackson
E. A. Burch, Jr. T. J. Friday

Introduction. – Although erythropoiesis and stem cell production have been extensively studied in mice, little is known about megakaryocytopoiesis. The maturation and differentiation processes in megakaryocytes of young adult male mice (RFM/Unf) were investigated using the method of determining the labeling index of several sequential morphologic stages or compartments of megakaryocytes at intervals after injection of tritiated thymidine. Three morphologic stages were distinguished: those megakaryocytes with clear, basophilic cytoplasm and nuclear chromatin having an evenly stippled appearance were classified as type I, the most immature stage of the recognizable population; those with acidophilic, granulated cytoplasm and nuclear chromatin having a moderately dense and uneven appearance were classified as type II cells, a more mature stage; the final stage, type III, was comprised of nuclei without cytoplasm. The results will provide a foundation for exploring the effects of age, radiation, and other factors on megakaryocytopoiesis, and for investigating the control mechanisms involved in its regulation.

Results and Discussion. – The frequencies of recognizable megakaryocytes among 26 mice were as follows: type I, 10% ± 2 (1 standard deviation); type II, 86% ± 3; type III, 4% ± 2. At 30 min after injection of ^{3}HTdr, about 24% of type I megakaryocytes were labeled but none of the more mature megakaryocytes. By 24 hr 100% of the type I megakaryocytes were labeled. Label was first seen in type II megakaryocytes at 4 hr, increased slowly to 10% at 18 hr, and then more rapidly, reaching its maximum of about 90% at approximately 48 hr. The first labeling of naked nuclei was seen at 48 hr, when about 30% were labeled. It continued to increase and reached nearly 100% by 72 hr. The appearance of label first in type I cells and later in types II and III indicates that these are indeed sequential differentiation compartments. The time relations and the changing rates of increase of the labeling index suggest that the time spent in the several stages may vary considerably among cells. The attainment of full labeling of mature megakaryocytes (type II) at about 50 hr indicates the duration of the total maturation time of recognizable megakaryocytes of these mice. Using the initial slope of the labeling rate curve of type I megakarycytes, about 15 hr are required to reach 100% labeling; this constitutes an estimate of the average time spent in the type I compartment. We estimate that 42 hr are spent in the type II compartment, using a rate curve drawn through the experimental points between 18 and 48 hr. Adding the latter two estimates gives a total maturation time through the type II stage of 57 hr, in reasonably good agreement with the 50 hr estimates from the time taken to reach full labeling.

18.9 EFFECTS OF X IRRADIATION ON MEGAKARYOCYTOPOIESIS IN MICE

T. T. Odell, Jr. C. W. Jackson
E. A. Burch, Jr. T. J. Friday

Introduction. – Since a lack of blood platelets is in large part responsible for the hemorrhagic syndrome that results from whole-body exposure to sublethal to lethal doses of radiation, an understanding of the effects of radiation on the maturation of megakaryocytes, the source of platelets, is needed. Therefore the effects of whole-body x irradiation on the maturation

of megakaryocytes in the marrow of young adult male mice (RFM/Unf) were investigated by determining the frequency of morphologic maturation stages at intervals during the 72 hr following exposure to 650 r of x rays. Three morphologic stages of megakaryocytes were distinguished, as described in the preceding report, type I megakaryocytes being immature, type II being mature, and type III being degenerating nuclei without cytoplasm.

Results and Discussion. – As a percentage of the total population of recognizable megakaryocytes, the type I megakaryocytes gradually declined from the control level of 9.3%, until at 72 hr they comprised 1.3% of the population. The type II megakaryocytes stayed within the control range of about 85 to 90% except at 48 hr, when they were relatively more abundant than normal. The percentage of naked nuclei (type III) was significantly greater than that of the controls at 60 and 72 hr. These results are consistent with the concept that the recognizable megakaryocytes that are present at the time of irradiation continue to differentiate and produce platelets but that the entry of new megakaryocytes into the population is curtailed.

18.10 EFFECTS OF THROMBOCYTOPENIA ON MEGAKARYOCYTOPOIESIS

T. T. Odell, Jr. T. J. Friday
C. W. Jackson Deborah E. Charsha

Introduction. – Various studies have indicated that the concentration of blood platelets in the peripheral circulation has a regulatory effect on the production of platelets from megakaryocytes. This study was undertaken to investigate the postulate that the increases in megakaryocyte number and in platelet production in thrombocytopenic rats are due both to an increased rate of entry of new megakaryocytes into the recognizable population and to a reduction in the maturation time of recognizable megakaryocytes. Thrombocytopenia was maintained by daily injection of platelet-specific antiserum, and the effects on megakaryocyte number, mitotic index, frequency of cells in maturation compartments, and labeling index of the maturation stages at intervals after injection of tritiated thymidine were examined. The tritiated thymidine was injected 24 hr after the first injection of antiserum, and two rats were killed at intervals during 72 hr. Megakaryocytes were classified as types I, II, or III according to the criteria described above.

Results and Discussion. – Twenty-four hours after the first injection of platelet-specific antiserum the megakaryocyte count in the marrow had begun to rise, and by 60 hr it had leveled out at a value more than twice that of controls. The mitotic index of the megakaryocytes (endomitosis) increased more than three times, reaching a peak around 36 hr after the first injection of antiserum and then declining. Both of these findings indicate that thrombocytopenia induces an increase in the rate of production of megakaryocytes. In addition, the frequencies as well as numbers of cells in the various stages of maturation varied with time, the most immature recognizable megakaryocytes becoming much more numerous by 36 hr after antiserum and the more mature stages increasing later. Moreover, the labeling index studies showed that the maturation rate of recognizable megakaryocytes was also increased, since the rate of appearance of labeled cells in the several compartments of the megakaryocyte population was more rapid in the thrombocytopenic rats than in controls. Thus thrombocytopenia causes both an increase in the rate of entry of cells into the recognizable megakaryocyte population and an increase in the maturation rate of those cells.

18.11 LIFE-SHORTENING EFFECTS OF X RADIATION IN GERMFREE MICE

H. E. Walburg, Jr. G. E. Cosgrove

Introduction. – It is well substantiated that x radiation reduces the life-span of mice, both by increasing the incidence of specific early-occurring diseases, such as thymic lymphoma, and by a "nonspecific" decrease in length of life. To determine if a decline in the effectiveness of defense mechanisms of the animal and a resultant increase in susceptibility to infectious diseases play a role in the "nonspecific" decrease in life-span, the life-shortening effects of x radiation were studied in germfree, as compared with conventional, mice.

Results and Discussion. – The results of two experiments on the late somatic effects of radiation were analyzed for evidence of life shortening: (1) male germfree and conventional 11- to 14-week-old ICR mice which survived whole-body exposure to 600–900 r of x rays and (2) male and female RFM mice, reared either in germfree or conventional environments, which were exposed at 5 to 6 weeks of age to 300 r of whole-body x radiation.

When compared with unirradiated controls, irradiated mice of all groups showed life shortening if all causes of death were considered (Table 18.11.1). The amount of life shortening varied from only 0.03 day/r in the case

Table 18.11.1. Life-Shortening Effect of X Radiation in Germfree Mice

Strain	Sex	Environment	Life Shortening (days/r)	
			All Causes	All Causes Other than Thymic Lymphoma, Myeloid Leukemia, Cecal Volvulus
ICR	♂	GF	0.16	0.34
		CONV	0.03	0.12
RFM	♂	GF	0.77	0.34
		CONV	0.43	0.16
	♀	GF	1.13	0.68
		CONV	0.96	0.68

of conventional ICR males to 1.13 days/r in the case of germfree RFM females. In general, germfree mice had greater life shortening than did their conventional counterparts, and the females had greater life shortening than did the males.

Thymic lymphoma and myeloid leukemia, which are early-occurring radiation-induced diseases, and cecal volvulus, which is seen in germfree mice owing to the characteristically enlarged cecum of such animals, differ widely in incidence in germfree, as compared with conventional, mice. If mice dying of these diseases are withdrawn from the sample under consideration, life shortening in the remaining mice more accurately reflects a "nonspecific" type of life shortening. Under these conditions, males of the two strains show a similar response to radiation, with life shortening being two to three times greater in the germfree mice than in their conventional counterparts. Female RFM mice show a greater life shortening than males, and no difference is observed between the germfree and conventional animals of this sex.

These data suggest that failure of the defense mechanisms, with subsequent infectious disease, is not a factor in radiation-induced life shortening. The greater susceptibility to life shortening characteristic of germfree males, as compared with conventional males, may not be observed in females because of overriding specific diseases induced by radiation in females, that is, ovarian or pituitary tumors. Further studies will be carried out to clarify this question and to explore whether the microbial flora reduces the life-shortening effects of radiation by increasing "stem cell" pool sizes, providing an essential nutrient, or by some other mechanism.

18.12 HEMOPOIETIC RECOVERY IN X-IRRADIATED GERMFREE MICE

H. E. Walburg, Jr. Edna I. Mynatt

Introduction. – Conventional mice exposed to x radiation and injected with antibiotics have a markedly increased 30-day survival when compared with their irradiated but uninjected counterparts, presumably due to control of postirradiation bacterial infection. It was predicted that germfree mice would have an even greater survival following exposure to x radiation in the $LD_{0/30}$ to $LD_{100/30}$ range when compared with their conventional counterparts. Results of such treatment, however, demonstrate only a small increase of the $LD_{50/30}$ in germfree mice. To determine if repopulation is deficient in irradiated germfree mice, organ incorporation of ^{125}iododeoxyuridine (^{125}IUdR), which has been shown to be an accurate estimate of hemopoietic organ repopulation, was measured in irradiated germfree and conventional mice.

Results and Discussion. – Hemopoietic recovery of the spleen, bone marrow, mesenteric lymph node, and thymus was studied at various times after exposure to 500 r of x rays in 16- to 20-week-old male or female CF No. 1 mice reared in germfree or conventional environments. In general, the recovery of hemopoietic organs of germfree mice was similar to that of their conventional counterparts, with depression and recovery of hemopoiesis occurring at about the same time after irradiation, independent of the microbial environment. On the other hand, the amount of IUdR incorporation was significantly lower in the spleens and mesenteric lymph nodes of germfree mice than in those of their conventional counterparts. No such difference was

noted in comparing the bone marrow and thymus incorporation of ^{125}IUdR in irradiated germfree and conventional mice. These data suggest that the microbial flora, although not influencing the rate at which repopulation occurs, does significantly alter the amount of radiation-induced compensatory elevation of hemopoiesis in the spleen and mesenteric lymph nodes.

The amount of hemopoiesis which occurs in the spleen, bone marrow, and mesenteric lymph node (but not thymus) of unirradiated germfree mice is consistently lower than that in the organs of their conventional counterparts. This reduced activity of hemopoietic cells may be related to the reduced hemopoiesis in repopulating spleen and mesenteric lymph nodes of irradiated germfree mice. Such a relation is supported by the reduced yield of endogenous spleen colonies in irradiated germfree mice. Additional studies will be conducted to clarify the capacity of the hemopoietic system of germfree mice to repopulate after exposure to x radiation.

18.13 HIGH LEUKEMOGENIC POTENCY OF CULTURE FLUID FROM LEUKEMIA-VIRUS-INFECTED CELL CULTURES

R. L. Tyndall J. A. Otten
N. D. Bowles A. C. Upton

Introduction. – Murine leukemia viruses propagated in established cell cultures generally exhibit low leukemogenic potency. Rauscher leukemia virus (RLV) propagated in an established bone marrow culture (JLS V10) was reported to have minimal leukemogenic potency. The leukemogenicity of culture fluid from a spleen-thymus cell culture (JLS V5) propagating RLV was found to be one ID_{50} per milliliter of 100X concentrate. Moreover, such "attenuated" virus from the 'V5 culture has been shown to be an effective vaccine in protecting mice against challenge with animal-passaged RLV.[1]

We have previously described a variant of the JLS V5 culture which differs from the corresponding uninfected control culture (JLS V6) in showing an increased mucopolysaccharide production and ability to induce myxofibrosarcomas in newborn BALB/c mice. From such a primary myxofibrosarcoma, a culture has been derived which also continuously propagates leukemia virus. Another cell culture (RZO) has also been established by infecting a cloned control JLS V6 cell culture with purified RLV virus (banded in a sucrose gradient from the variant JLS V5 culture fluid). Since RLV which propagated in the original untransformed JLS V5 cultures was reported to be virtually nonleukemogenic,[1] studies were undertaken to determine if the virus propagated in the transformed JLS V5 cells and derived cultures showed an increased malignant potential.

Results and Discussion. – Culture fluid from the JLS V5 variant and derived cultures was either filtered through 0.45-μ filters, which were checked for cell exclusion with *E. coli*, or subjected to 4000 r irradiation. Serial tenfold dilutions of such culture fluids were made, and 0.1 ml of the fluid was injected intraperitoneally into one- to seven-day-old BALB/c mice. The mice were observed for a period of 13 months. It is evident from Table 18.13.1 that relatively high leukemogenic potency is associated with fluid from all three cultures. Unlike typical spleen-passaged RLV infections, however, the leukemias resulting from injection of these culture fluids were classified as reticulum-cell sarcomas or lymphomas with little or no associated erythroblastosis. Latent periods from the time of injection to the detection of overt leukemias ranged

Table 18.13.1. Leukemogenic Potency of Culture Fluids from Different RLV-Infected Cell Cultures

Dilution of Culture Fluid Injected	Cultures Tested, Incidence of Leukemias Induced,[a] and Average Latency (months) Before Onset of Overt Leukemia		
	Transformed JLS V5	RZO	Myxofibrosarcoma
Undiluted	7/8 (3 months)	8/8 (5 months)	4/4 (4 months)
1/10			4/4 (4 months)
1/100	17/18 (8 months)	15/18 (8 months)	22/23 (5 months)
1/1000		2/2 (10 months)	19/21 (6 months)
1/10,000	5/10 (13 months)		19/23 (6 months)

[a]Number of mice developing leukemia/number injected.

from 3 to 13 months. Leukemias routinely developed in animals inoculated with $^1/_{100}$ dilutions of fluids from either the variant JLS V5, myxofibrosarcoma-derived, or RZO cultures. No leukemias were observed in 130 uninoculated control animals housed in the same animal room for comparable time periods or in 20 animals inoculated with culture fluid from the uninfected, control JLS V6 cells.

The use of one- to seven-day-old test mice and the subsequent holding of such animals for a 13-month observation period is the most probable explanation for the ostensibly higher potency of culture fluid from our variant of the JLS V5 culture, as compared with that reported for the original untransformed 'V5 culture. Support for this explanation is indicated in additional studies now being carried out to determine the leukemogenic potency of fluids from the original untransformed JLS V5 culture and from the JLS V10 cell culture.

Preliminary results of these experiments indicate leukemogenic activity ($\geqslant$100 ID_{50} per milliliter of culture fluid) is detectable in fluids from either untransformed JLS V5 or from JLS V10 culture when inoculated into newborn BALB/c mice held for extended observation periods. More complete results of these ongoing studies will be given in future reports. Such data indicate, however, that inoculation of newborn mice subsequently followed for a prolonged observation period and lack of virus pelletization probably account for the differences observed to date. Further studies are presently under way, testing the leukemogenic activity of leukemia viruses from a variety of infected cell cultures, in the hope of revealing the full potentials of such viruses.

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18.14 THE REJECTION OF TRANSPLANTABLE SARCOMAS INFECTED WITH THE RAUSCHER LEUKEMIA VIRUS

R. L. Tyndall J. A. Otten
A. C. Upton

Introduction. – We have previously described the origin of a transplantable myxofibrosarcoma, infected with Rauscher leukemia virus (RLV), following subcutaneous injection of newborn BALB/c mice with cultured JLS V5 cells infected with RLV. Inoculation of the corresponding control uninfected cell culture (JLS V6) produced a transplantable spindle cell sarcoma free of infectious leukemia virus. It was observed that mice passively immunized with RLV vaccine were protected against challenge with the myxofibrosarcoma cells.[1] No such protection was evident in immunized mice against either the spindle cell sarcoma or tumors induced by daily administration of diethylnitrosamine. The present study was undertaken to determine the antigenic specificity and scope of sarcoma rejection in mice immunized with leukemia virus vaccines.

Results and Discussion. – In order to test the antigenic specificity of the myxofibrosarcoma rejection, vaccines were prepared from murine leukemias of varying etiologies, such as spontaneous thymic lymphoma tissue of AKR mice, leukemic spleen extract from mice infected with the Friend leukemia virus (FLV), leukemic spleen extract of rats infected with RLV, and pelleted RLV virus and resultant virus-free supernates from extracts of leukemic spleen tissue of mice infected with RLV. Newborn mice of mothers immunized with one of the various vaccines were challenged with myxofibrosarcoma cells. Such mice passively immunized with RLV vaccine prepared from rat tissue, vaccine prepared from pelleted RLV or the corresponding virus-free supernate, and vaccine prepared from RLV were protected against challenge with myxofibrosarcoma cells. Mice passively immunized with the leukemic AKR vaccine (Gross antigen) showed no such protection.

In order to determine if another sarcoma would also be amenable to such rejection, a transplantable spindle cell sarcoma infected with RLV was obtained following the inoculation of newborn BALB/c mice with an established bone marrow culture (JLS V10) transformed by infection with RLV. Inoculation of the corresponding uninfected control culture (JLS V9) produced a transplantable spindle cell sarcoma free of RLV. When newborn mice of mothers immunized with RLV vaccine were challenged with RLV-free spindle cell sarcomas (V9 tumors), no antitumor effect was noted. Conversely, complete protection against tumor growth occurred in mice challenged with spindle cell sarcomas infected with RLV (V10 tumors) at cell doses producing 100% incidence in nonimmunized control animals.

The results thus far indicate that the antigen(s) involved in the rejection of RLV-infected sarcomas are of the FMR (Friend, Moloney, Rauscher) group. The data also indicate that sarcomas other than the myxofibrosarcomas can be rejected in mice immunized against the FMR antigens, provided that the tumor is infected with RLV leukemia virus or presumably with

any of the FMR group viruses. Moreover, the effective antigenicity of the virus-free soluble protein vaccine made from leukemic spleen cell extract indicates that the antigen(s) involved in the tumor rejections would be amenable to purification and identification. The results also demonstrate the feasibility of using tumor rejection to investigate the antigenic relationship of various spontaneous or virus-induced murine leukemias. Studies are presently under way to test the effectiveness of vaccines prepared from various leukemic tissues in inhibiting the growth of RLV-infected sarcomas as a measure of the antigenic relationship between human and murine leukemia antigens.

Reference

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18.15 IMPROVED HIGH-RESOLUTION ELECTROPHORESIS IN ACRYLAMIDE GELS EMPLOYING A NEW CONSTANT POWER SUPPLY AND GRADIENT FLAT-BED GELS

R. C. Allen R. H. Dilworth[1]

Introduction. – A major factor limiting resolution when electrophoretic separations are performed by constant voltage or constant current is the effect of Joule heating within the electrophoresis cell, which, if excessive, will cause convective mixing and diffusion of the components to be separated, both of which result in a loss of resolution, with the latter effect depending on time and temperature. The disk technique developed by Ornstein and Davis, in which the sample is gelled in an anticonvective large-pore-size medium, obviates the problem of convective mixing in the sample and is suitable for routine protein separations; however, in certain isoenzyme separations performed with this technique, there is a marked loss of activity during the sample gelling process, and the sample is best placed in a convective medium such as sucrose for accurate quantitative studies. As a result, lower currents must be employed to prevent inactivation of heat-labile isozymes. Thus the effective separation time is prolonged, with a resulting decrease in resolution, owing to increased diffusion and occasionally to convective mixing of the sample, which is not always corrected in the process of stacking in the sucrose layer. As a result of the forced compromise in selection of operating voltage or current to accommodate these limiting factors, present electrophoretic processes provide less than optimal resolution.

Results and Discussion. – To overcome some of the limitations imposed by constant-current or constant-voltage power supplies, the following four techniques have been developed for use in combination to improve resolution, three of them involving the power delivery to the cell and the fourth involving the gel pore size and electrochemical properties of the system:

1. The electric field is applied to the cell with regulation of the power supply so arranged as to deliver constant power to the cell rather than constant voltage or current.
2. This constant power is delivered in the form of pulses having a low duty cycle, rather than as direct current, thus greatly reducing Joule heating.
3. The regulated power is programmed to vary in a chosen manner during the time period of the separation.
4. The conductivity-shift procedure of Hjerten is combined with the trailing-leading ion front and associated zone-shaping effects resulting from voltage gradient changes to afford further improvement of resolution.

In addition, to take fuller advantage of the sieving effect of acrylamide gel, gradient gels are employed which provide pore sizes compatible with maximal resolution of both high- and low-molecular-weight plasma protein components.

With the innovations described in this report, the resolution of proteins and esterase isoenzymes has been significantly improved, and the time required for a separation has been reduced simultaneously by a factor of 2 or more.

Reference

[1] ORTEC, Inc., a subsidiary of Edgerton, Germeshausen & Grier.

18.16 SPLENIC FIBRINOSIS IN MOUSE RADIATION CHIMERAS WITH SECONDARY DISEASE

W. D. Gude C. C Congdon[1]
G. E. Cosgrove

Irradiated mice that have received allogeneic bone marrow, parental spleen, or parental bone marrow cells soon develop a clinical syndrome called "secondary disease," or "foreign spleen or bone marrow reaction." Considered to be an immunologic reaction, the disease is characterized histologically by extensive lesions in the lymphatic tissues (lymph nodes, white pulp of spleen,

Table 18.16.1. Relationship of Splenic Fibrinoid Necrosis to Radiation Dose and Types of Cells Injected in Pooled Mice of All Strains

Radiation Dose (rads)	Type of Cells Injected	Fibrinoid Necrosis			
			Severity		
		Frequency	Mild	Moderate	Severe
300–900	None	0/91			
0	Parental spleen or bone marrow[a]	0/30			
400–900	Isogeneic spleen or bone marrow	2/32	2		
900	Parental bone marrow[a]	6/114	2	3	1
400–900	Parental spleen[a]	44/402	21	6	17
900–950	Allogeneic bone marrow	31/94	1	19	11

[a]Parental donor cells injected into F1 hybrid recipients.

and Peyer's patches), along with associated atrophy of thymus, bone marrow, red pulp of spleen, and other organs. In varying incidence, the lesions in lymphatic tissues are associated with areas of fibrinoid necrosis (Table 18.16.1). This report was an attempt to study the histochemical nature of the fibrinoid lesions in the spleen accompanying secondary disease.

Spleen lesions from three strains of mice were studied by means of five histochemical stains commonly used to demonstrate fibrin. Threadlike deposits within the lesions reacted with these stains, revealing the presence of fibrin. However, intervening areas in the lesions did not react with fibrin-specific stains. The morphological and other staining characteristics of the necrotic lesions were, however, identical with those generally described in fibrinoid necrosis.

Interpreting these findings as representative of one type of fibrinoid necrosis and not of fibrin alone, we agreed with the hypothesis that fibrinoid was formed from either plasma proteins or as a terminal step in the sequence of fibrinogen degeneration in areas of necrotic ground substance.

Reference

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19. Cell Growth and Differentiation

Tuneo Yamada

Control of Cell Differentiation (19.1–19.10)

Tuneo Yamada
D. S. McDevitt[a]
R. K. Achazi[b]
S. P. Modak[a,b]
Rolf Nöthiger[b]
M. Virginia Cone
Lola M. Kyte
D. H. Reese
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Betty Jean Persons[d]

Chromosome Ultrastructure (19.11)

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Barbara R. Beatty
J. R. Palisano[d]

Vitellogenesis (19.12–19.17)

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19.1 ULTRASTRUCTURAL ALTERATION OF NUCLEOLI, CHROMATIN, AND CORTEX OF IRIS CELLS IN INDUCTION OF CELL PROLIFERATION

Tuneo Yamada J. N. Dumont
M. Virginia Cone

Lentectomy of the adult newt eye induces DNA synthesis and subsequent cell replication in the iris epithelium of the operated eye. This induction of proliferation is interesting, not only because it leads to dedifferentiation and redifferentiation, but also because it occurs in a tissue in which normally no DNA synthesis can be detected. Under our experimental conditions, induced DNA synthesis becomes observable four days after lens removal.[1] In the present study, we are interested in the possible cellular changes which occur before induced DNA synthesis. Ultrastructure of cells of the dorsal iris epithelium two and four days after lens removal was compared with that of the normal iris epithelium. Definite alterations are found in ultrastructure of nucleolus, chromatin, and cell cortex.

Nucleolus. – The normal iris epithelial nuclei contain small groups of the condensed fibrous component embedded in the chromatin. These areas are identified as "inactive nucleoli." In the two-day iris epithelium, in addition to those "inactive nucleoli," large nucleoli can be observed which are composed of a zone of granular components and a zone of fibrous components. This type of nucleolus, which is known to be associated with active RNA synthesis, represents the majority of nucleoli found in the four-day regenerate. Thus transformation of the nucleolus seems to start within two days after lens removal, before the induced DNA synthesis starts, and subsequently affects the increasing number of nucleoli present. The results are in good agreement with the observation that ribosomal RNA synthesis is activated in the iris two days after lens removal.[2]

Chromatin. – In the normal iris nuclei the chromatin area with the condensed chromatin fibrils predominates

over the interchromatin area with its dispersed chromatin fibrils. In the two-day series the interchromatin area increases at the expense of the chromatin area. A further expansion of the interchromatin area is indicated in the four-day series. These intranuclear changes are associated with a change in the shape of the nucleus: the numerous indentations of the nuclear membrane, which characterize the normal iris epithelial cells, gradually disappear, and the nucleus assumes a more or less ellipsoidal shape. It is probable that these alterations are correlated with the enhancement of RNA synthesis demonstrated by autoradiographic[3] and biochemical methods.[2]

Cell Cortex. – In contrast to the external (apical) surface of the normal epithelial cell, which is poorly organized and consists of a thin irregular layer of cytoplasm which is often interrupted by pigment granules and cytoplasmic membranes, the corresponding area of the four-day cell is a regular layer of 0.2 to 0.3 μ thickness of compact cytoplasm. Pigment granules, mitochondria, cytoplasmic membranes, and ribosomes, all of which are abundant in the main part of cytoplasm, are almost absent in this layer. The appearance of the external cortical layer is accompanied by close packing of iris cells in the epithelial arrangement and suggests an increase in homotypic cell affinity. This cytoplasmic alteration is hardly observed in the two-day series.

Contributions of the present work to the understanding of the mechanism of activation of cell proliferation can be summarized in the following way: (1) Morphological transformation of the nucleolus precedes the entrance of cells into the induced S phase. (2) An unsuspected cytoplasmic change at an early phase of tissue transformation has been detected. (3) In the time sequence the first detectable morphological change in the cytoplasm follows that in the nucleus.

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19.2 INITIATION OF DNA SYNTHESIS DURING INDUCED CELL PROLIFERATION IN THE NEWT IRIS EPITHELIUM

Tuneo Yamada Marion E. Roesel

In the past a few papers have been published from our group on the pattern of DNA synthesis in Wolffian lens regeneration.[1,2] For our recent efforts[3,4] to understand the mechanism involved in the induction of cell proliferation by lens removal in the newt iris epithelium, some specific information on the pattern of DNA synthesis in this system is needed which is not covered by earlier works. The questions are: (1) How complete is the suppression of DNA synthesis in the iris epithelium in the normal condition? (2) On which day after lens removal does the induced DNA synthesis become detectable in the iris epithelium? (3) Is there any localization of cells in the S phase within the iris epithelium at a given time? The present experiments are designed to answer these questions.

Normal adult newts (*Triturus viridescens*) and adult newts one, two, three, four, and five days after unilateral lentectomy were injected intraperitoneally with 1 μc per gram of body weight of ^{3}H-thymidine. Two hours later their heads were fixed, and the iris was processed to obtain complete serial sections of 3 μ thickness with histological orientation for autoradiography. All observable nuclear sections in the whole iris epithelium proper, excluding those in pars ciliaris, stroma, and ora serrata, were scanned. The minimum number of silver grains per nuclear section to be evaluated as labeled was decided for each group of slides on the basis of grain counts of nuclear sections of cornea epithelium which was included in sections.

The data so far obtained show that (1) labeled cells cannot be detected at all in the iris epithelium of normal animals or of nonoperated eyes of unilaterally operated animals; (2) labeled cells start to become detectable four days after lens removal in the iris epithelium of operated eye; (3) in the series with labeled cells, no definite localization of labeled cells either in the dorsal, lateral, or ventral sectors of iris epithelium can be found. Neither is labeling restricted to either the external or internal lamina of the iris epithelium. However, the possibility of variations in frequencies of labeled cells in those different compartments are not excluded. (4) Stroma, ora serrata, and the macrophage population surrounding the iris epithelum indicated labeled cells in all series.

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19.3 FURTHER STUDIES OF RIBOSOMAL RNA SYNTHESIS DURING TISSUE TRANSFORMATION

D. H. Reese Tuneo Yamada

In continuation of the work[1] reported earlier on the sedimentation pattern of RNA synthesized by the tissue population involved in Wolffian lens regeneration, some results were obtained which appear to be pertinent for understanding the mechanism of induction of cell proliferation:

1. A number of dorsal marginal irises of the normal adult newts or of adult newts at various time intervals after lens removal were isolated and labeled in an amphibian culture medium containing 2μc/ml ^{14}C(U)-uridine for 5 hr, and the homogenate was extracted after addition of the rat liver RNA as a carrier with phenol at 4°C. The sedimentation profile of radioactivity was studied on the sucrose density gradient. The comparison of the patterns shows that in the normal condition, labeling of RNA is insignificant except in the 4S region, whereas starting from two days after lens removal a significant labeling occurs in the 28S and 18S regions. The extent of labeling in these regions increases from two to ten days after lens removal and remains at a high level throughout regeneration.

2. When phenol extraction was done at 60°C, after the same labeling experiment, and the extracted RNA was studied on the sucrose gradient, high incorporation was found in the area between the bottom and 28S region as well as in other areas in all stages of lens regenerates studied. But in the normal iris, incorporation in the former area is insignificant as well as in other areas.

Assuming that a change in the precursor pool is not obscuring the present data, the results reported in item 1 show that synthesis of 28S and 18S RNA is activated by lens removal and that this activation starts two days after lens removal. The small amount of material available does not allow further characterization of both RNA's. However, that they represent the two larger rRNA's is suggested by the fact that the nucleolus transforms from the inactive form into the active form[2] at the time those two RNA's begin to be synthesized, and soon thereafter the cytoplasmic ribosome population increases. Thus the present data make probable that production of 28S and 18S rRNA's is controlled by lens removal. Theoretically this can be done either by affecting transcription of the ribosomal RNA precursor or by affecting its transformation into both rRNA's. A review of the present data indicates that the precursor, which has a sedimentation coefficient of 40S in the amphibian cells,[3] is extracted under the present condition with the hot phenol but not by cold phenol. In item 2, incorporation into the 40S region is insignificant in the normal iris but is highly significant in all lens regenerates studied. This makes probable that rRNA synthesis is controlled at the transcriptional rather than at the posttranscriptional level in this system. It should be further noted that the activation of ribosomal RNA synthesis is the first definite cellular event so far found after lens removal and precedes the induced DNA synthesis[4] and cell replication.

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19.4 RETENTION OF MELANIN BY IRIS CELLS IN CULTURE

D. H. Reese

We reported previously[1] that 0.25% trypsin dissociation of dorsal iris epithelium results in depigmentation of the disociated cells when put into culture. Furthermore, the dissociated cells actively proliferate and have continued to do so for over 16 months. Depigmentation and activation of cell division in the iris both are events characteristic of Wolffian lens regeneration. Recently we have found conditions which allow iris cells to retain their pigment in culture for extended periods of time. By dissociating dorsal iris tissue in 0.1% trypsin and 0.04% EDTA, iris cells dissociate into individual cells and clusters of cells in *ca.* 1 hr. In culture these cells attach to the surface of the dish but do not spread out for almost a week; however, they retain their pigment granules. The morphology of these cells is quite different from iris cells dissociated in 0.25% trypsin. If established cultures of iris cells[1] are grown in culture medium for six days, the medium becomes "conditioned." After filtration to remove any cells in suspension, this "conditioned" medium greatly enhances the ability of dissociated pigmented cells to attach and spread out.

Reference

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19.5 ONTOGENY OF LENS-SPECIFIC PROTEINS IN EMBRYONIC AND LARVAL (METAMORPHIC) *RANA PIPIENS*

D. S. McDevitt

By use of immunofluorescent techniques, it has recently been demonstrated in this laboratory[1] that lens-specific proteins are first detectable in the lens rudiments of *R. pipiens* embryos at the time of elongation of those cells comprising the internal layer of the lens vesicle. Anti-total lens protein fluorescent antibody and anti-γ crystallin fluorescent antibody are both positive for their respective antigens at this stage. The localization of the immunofluorescence reaction for the former is in the above area of cell elongation and, with lesser intensity, in the external layer. Fluorescence due to the anti-γ crystallin antibody is limited to this region of cell elongation and, as lens development progresses, remains restricted to the lens fiber area.

In an attempt to determine the antigenic basis for the observed difference in cellular localization of the two fluorescent antibody preparations, lenses of Shumway stage 25 and Taylor-Kollros stages I, VI, and XXIV *R. pipiens* were removed microsurgically, and total lens protein samples of each stage were prepared. When such samples were subjected to immunoelectrophoretic analysis, using absorbed adult anti-total lens protein antibody, only a single precipitin arc corresponding to γ crystallin was readily apparent at Shumway stage 25. In the metamorphic Taylor-Kollros stages I and VI a β-crystallin component can first be seen to appear, and at Taylor-Kollros stage XXIV a component with α-crystallin mobility can be observed, and the patterns of adult and stage XXIV total lens proteins are identical. Ouchterlony analysis extended these observations in that both a γ-crystallin *and* a β-crystallin precipitin line cannot be detected until Taylor-Kollros stage XXIV. Thus, within the limits of the techniques employed, this investigation indicates that γ crystallin, indicative of lens fiber differentiation in this organism, is one of the first lens-specific proteins to be detected, while α crystallin is the last of the crystallins to appear, late in *R. pipiens* lens development.

Reference

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19.6 ENTRANCE OF PROSPECTIVE PRIMARY FIBER CELLS INTO THE TERMINAL CELL PHASE IN THE NORMAL DEVELOPMENT OF THE FROG LENS

Tuneo Yamada D. S. McDevitt
Marion E. Roesel

In the newt lens regenerating system the lens-specific proteins first become detectable in the prospective primary fiber cells, when they complete the final S phase and enter the terminal cell phase.[1,2] The question has been raised whether or not a similar situation applies to the normal lens development. Appearance of lens-specific proteins, especially gamma crystallins, in the cells of the normal lens rudiment in *Rana pipiens* has been studied with immunofluorescence.[3] The data indicated that these crystallins first become detectable at the stage of lens vesicle when it has been separated from the ectoderm. The elongating prospective primary fiber cells, which are located in the internal wall of the vesicle, become positive at this time. Those cells, like all other cells of the vesicle, are derived from the head ectoderm and have been in the cell cycle since fertilization. Our question then is the temporal relationship between exit of those cells from the cell cycle and their acquisition of lens specificity. In the present study the timing of the final S phase of the prospective primary fiber cells was studied with autoradiography under the condition that the majority of cells in the cell cycle become labeled either in vitro or in vivo.

In the in vitro experiment the head region of *Rana pipiens* embryos was surgically isolated at various developmental stages ranging from late neurula to late tail bud and cultured in L-15 culture medium which was adjusted for amphibia and contained 5 μc/ml of ^{3}H-thymidine. After continuous labeling for 48 hr, during which the medium was renewed once, the explants were fixed and processed for autoradiography in sections. In the in vivo experiment the embryos of various tail bud stages were injected with 0.005 μc per embryo every 10 hr from the beginning of a 48-hr period. After this period the embryos were fixed and processed as above. In both groups of experiments, tissues were classified according to the lens developmental stage[3] at which the labeling started. Series 5, for instance, includes tissues labeled from lens developmental stage 5 on for 48 hr.

Using autoradiographs obtained in this way, the percentage of nonlabeled cells in the prospective pri-

mary fiber area was obtained for various series of the in vitro group. Nonlabeled cells were absent in series 1 and 2, and started to appear in series 3. Their percentage increased from 13 to 98 between series 4 and 5 and reached 100 in series 6. From these data one can conclude that the prospective primary fiber cells complete their final S phase during lens developmental stages 3 to 5 and subsequently enter the terminal phase. That they divide after the final S phase is suggested by frequent occurrence of mitotic figures in the corresponding area of the lens rudiment at the corresponding stages. The in vivo series gave closely comparable results.

When these data are related to the immunofluorescence data for the same system, it appears very probable that the prospective primary fiber cells become positive for gamma crystallins soon after completion of the final S phase.

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19.7 TERMINAL LENS CELL DIFFERENTIATION: III. PRIMING ACTIVITY OF DNA DURING NUCLEAR DEGENERATION

S. P. Modak F. J. Bollum[1]

It has been shown previously in this laboratory that during the terminal phase of cell differentiation, lens fiber cell nuclei degenerate and disappear.[2] During the nuclear degeneration, a relative amount of Feulgen positive material or 2650-A absorbing nucleic acids gradually disappears.[2] Analysis of the template activity of DNA in lens cell nuclei[3] reveals that DNA does not become appreciably denatured during the degenerative process. On the other hand, acid denaturation reveals a relatively higher susceptibility of DNA in the degenerating fiber cell nuclei, which act as better templates for calf thymus DNA polymerase than healthy fiber or epithelial cell nuclei, probably due to a large-scale strand breakage.[3] In this report we have, for the first time, introduced the use of deoxynucleotidyl terminal transferase for the detection of much smaller single-stranded regions or fragments of DNA in lens cell nuclei.

Alcohol-fixed lenses were embedded in paraffin and sectioned. Two-micron-thick sections were mounted on a coverslip which was attached to an incubation well. Sections were incubated with deoxynucleotidyl terminal transferase and substrates containing either ^{3}H-dATP or ^{3}H.TTP. Control sections were pretreated with DMSO, 0.001 *N* NaOH, 0.01 *N* HCl, or heat followed by quick cooling, in order to denature DNA. Treated preparations were thoroughly washed in ice-cold 5% TCA [1% PPi]. Slides were coated with NTB-3 liquid emulsion, exposed for 24 hr, and developed in D.11 developer.

Examination of autoradiographs revealed that, without any denaturation procedure, degenerating fiber cell nuclei incorporated a significant amount of radioactivity. Probably the most interesting finding is that the central area of lens fibers contains highly localized incorporation of deoxynucleotide trisphosphate, although cell nuclei have completely disappeared from this region. In preparations treated for DNA denaturation, all types of cell nuclei incorporated radioactivity and showed the persistence of the nonnuclear incorporation in the center of the lens fiber area. Since terminal transferase can recognize much smaller fragments of polydeoxynucleotides than the DNA polymerase, we conclude that during the nuclear degeneration, there indeed occur free single-stranded ends (at least three nucleotides long) which serve as primers for the end-addition enzyme. Similarly, the presence of highly localized incorporation in the center of the lens fiber area, which lacks cell nuclei, demonstrates the presence of many small single-stranded fragments of DNA from the nuclei which have previously disappeared.

It would then appear from the above data and our previous work[3] that during nuclear degeneration, DNA undergoes strand breaks, resulting in free 3′ OH ends. This technique offers a great potential for localization of very minute amounts of single-stranded DNA and small fragments of single-stranded polydeoxynucleotides at the cellular and subcellular level. It should equally prove as a valuable tool for the detection and analysis of the nature of radiation-induced damage to DNA at the cellular level.

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19.8 THE PATTERN OF DNA SYNTHESIS IN THE LENS EPITHELIUM AND THE ANNULAR PAD DURING THE DEVELOPMENT AND GROWTH OF CHICK LENS

Betty Jean Persons S. P. Modak

In previous reports[1,2] we have shown that during the early morphogenesis of chick lens, the prospective lens epithelium retains cellular replicative activity at a relatively steady level, while cell division gradually decreases and disappears in the prospective fiber area. It was also found that cell division decreases in the presumptive annular pad region. In this communication, we are presenting the results obtained on the pattern of cell replication in these areas of older chick lenses.

Embryonic and postembryonic chick lenses were cultured in Minimum Essential Medium and Waymouth MB 752/1 supplemented with 15% chicken serum. Cultures were exposed to 2 μc/ml of ^{3}H- or ^{14}C-thymidine for 2 hr and immediately fixed in Carnoy's fixative. Autoradiograms of serially sectioned lenses were prepared. Lens epithelium and the annular pad were divided into five regions, namely: (1) central epithelium, (2) preipheral epithelium, (3) outer, (4) middle, and (5) inner annular pad. Numbers of DNA-synthesizing and total cells were estimated for different areas.

Analysis of the data reveals that, with development, overall DNA synthetic activity decreases in the entire external area. Cell replication becomes progressively localized to the peripheral epithelium and the outer region of the annular pad. The central epithelial cell population enters a stationary phase, while the middle and inner annular pad regions enter the terminal cell cycle. Although cell division ceases completely, actual cell number increases rapidly in the middle and inner regions of the annular pad. This fact, coupled with the localized cell replication in the outer annular pad and the peripheral epithelium, is interpreted as a progressive attainment of a specific growth pattern controlling the displacement of epithelial and annular pad cells into the lens fiber area.

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19.9 THE PATTERN OF RNA SYNTHESIS IN DEVELOPING CHICK LENS

S. P. Modak Betty Jean Persons

Eight- and fourteen-day embryonic chick lenses were dissected out and put into organ culture using M.E.M. or Waymouth MB 752/1 defined media. Culture media were supplemented with 15% chicken serum. Cultures were exposed to 2 μc/ml of ^{3}H-uridine for 2 hr. At the end of incubation, the lens epithelium, the annular pad, and the lens fiber areas were dissected and separately squashed. Squashes were fixed in ice-cold Carnoy's fixative. Preparations were routinely treated, and autoradiograms were prepared by coating the slides with NTB-3 liquid emulsion and exposing for seven days. A few lenses were serially sectioned and autoradiographs prepared. Grains per nucleus from various regions were counted. The data revealed that with development, RNA synthesis gradually decreases in the central epithelium and reaches fairly low levels by 14 days of development. Highest incorporation of ^{3}H-uridine is found in the peripheral epithelium and the annular pad. Progressively decreasing levels of RNA synthesis are found during differentiation of lens fiber cells. RNA synthesis was absent in the primary fiber cell nuclei in a 14-day lens, indicating that nuclear degeneration[1] is preceded by a halt in RNA synthesis. Our results generally support earlier published observations,[2] except in one important detail. Contrary to the earlier report,[2] decreasing levels of ^{3}H-uridine incorporation are seen in the central epithelium. This suggests that as central epithelial cells enter a stationary phase in cell cycle,[3] a decrease in RNA synthesis occurs.

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19.10 A PRELIMINARY REPORT ON MATHEMATICAL FUNCTIONS TO PREDICT THE NUMBER OF HEALTHY AND DEGENERATING FIBER CELL NUCLEI IN DEVELOPING CHICK LENS

V. R. R. Uppuluri[1] W. E. Lever[1] S. P. Modak

Previous reports from this laboratory show that during the development and differentiation of chick

lens, fiber cell nuclei degenerate and disappear.[2,3] Nuclear degeneration and disappearance follow a strict temporal and spatial pattern.[3] Since the lens fiber cell population becomes nonreplicative at a very early embryonic stage,[4] any increase in the fiber cell population must be due to a displacement of annular pad cells into the fiber area.[5] We have observed that the replicating cell populations, namely, lens epithelium and the annular pad themselves, establish a specific growth pattern.[5] It is of interest to understand the relative behavior of the replicative and nonreplicative cell population in order to elucidate the nature of different parameters affecting lens growth and maturity.

In this report, we have examined a few mathematical functions used to predict the behavior of healthy and degenerating fiber cell nuclear populations. Thirteen chick lenses of various developmental stages were fixed and routinely processed to obtain serial sections. From the median sections, number of healthy and degenerating fiber cell nuclei were counted and corrected by Abercrombie's formula.[6] Assuming that fiber cell nuclei lie over a circular area along the dorsoventral or horizontal plane of the lens, total number of nuclei was estimated. Different mathematical functions were fitted to the data. In each case the models were fitted to the natural logarithm of the observed number of nuclei.

Models for Healthy Fiber Cell Nuclei. – The first model to be considered is

$$\ln N(t) = A\,[1 - \exp(-Bt^C)]\ , \qquad (1)$$

where $N(t)$ represents the number of healthy fiber nuclei present in a chick lens at developmental age t. Based on data from 13 chick lenses, values of A, B, and C are estimated. Thus:

$\hat{A} = 12.4476$	$\hat{S}_{\hat{A}} = 0.08882$
$\hat{B} = 0.396435$	$\hat{S}_{\hat{B}} = 0.02601$
$\hat{C} = 0.765648$	$\hat{S}_{\hat{C}} = 0.06122$

The observed values of $N(t)$ and the predicted values of $N(t)$ based on (1) and estimates of A, B, and C are given in Table 19.10.1.

The second model considered appears to be the best fitting model. This model calls for the division of the developmental age span into three groups. On each of these groups, ln $N(t)$ would be approximated by a straight line. These lines will have to be restrained so that they will intersect at the group end points. At this time parameter estimation has not been attempted.

Table 19.10.1. Observed and Predicted Healthy Nuclei

Day	Nuclei	
	Observed	Predicted
4.83	9173	9274
5	11694	10411
6.17	18656	20462
7	30106	29834
8	38595	43184
9	55710	58175
11	91633	90348
12	115943	106353
13	124409	121722
14	153351	136180
17	168038	172826
20	189340	199362
25	220705	226369

Models for Degenerating Fiber Cell Nuclei. – Presently, only a polynomial model has been considered for relating time to total degenerating nuclei. The data seem to indicate that a polynomial of the third degree will give the most adequate fit. Thus

$$\ln M(t) = \alpha + \beta t + \gamma t^2 + \delta t^3\ , \qquad (2)$$

where $M(t)$ represents the total number of degenerating nuclei present in a lens at developmental age t. Based on data from eight lenses, estimated values of α, β, γ, and δ are as follows:

$\hat{\alpha} = -14.4802$	$\hat{S}_{\hat{\alpha}} = 2.841$
$\hat{\beta} = 3.51077$	$\hat{S}_{\hat{\beta}} = 0.555476$
$\hat{\gamma} = -0.163294$	$\hat{S}_{\hat{\gamma}} = 0.0344564$
$\hat{\delta} = 0.00247553$	$\hat{S}_{\hat{\delta}} = 0.000677135$

The observed values of $M(t)$ and the predicted values of $M(t)$ based on (2) are given in Table 19.10.2.

Models for Total Fiber Cell Nuclei. – At present, only one model has been considered for relating time to the total number of nuclei; this model is the same as (1). Based on the data from 13 chick lenses, the estimates of A, B, and C are as follows:

$\hat{A} = 12.6168$	$\hat{S}_{\hat{A}} = 0.106955$
$\hat{B} = 0.394315$	$\hat{S}_{\hat{B}} = 0.0278824$
$\hat{C} = 0.750331$	$\hat{S}_{\hat{C}} = 0.0680083$

The observed total nuclei and the predicted total nuclei based on (1) and from the values of A, B, and C are given in Table 19.10.3.

Table 19.10.2. Observed and Predicted Degenerating Nuclei

Day	Nuclei	
	Observed	Predicted
4.83	0	
5	0	
6.17	0	
7	0	
8	0	
9	330	297
11	1660	2153
12	4784	4503
13	7443	8119
14	16199	12809
17	25976	26195
20	25907	27501
25	20443	20143

Table 19.10.3. Observed and Predicted Total Fiber Nuclei

Day	Nuclei	
	Observed	Predicted
4.83	9173	9206
5	11694	10338
6.17	18656	20423
7	30106	29952
8	38595	43728
9	56040	59469
11	93293	94208
12	120727	111982
13	131852	129373
14	169550	146035
17	194014	189745
20	215247	222999
25	241148	258823

On the basis of the results obtained, it is concluded that the fits obtained by using the mathematical functions considered in this report are adequate. Accordingly, we are now in process of collecting extensive data on healthy and degenerating fiber cell nuclei at various stages of development, growth, and aging in chick lens. It is hoped that by a detailed analysis of the population behavior in lens fiber area, we would be able to estimate the fiber cell population which continues to become deprived of cell nuclei. Similarly, new models are being sought for describing the behavior of epithelial and annular pad cell population.

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19.11 EXTRACHROMOSOMAL NUCLEOLAR GENES IN AMPHIBIAN OOCYTES

O. L. Miller, Jr. Barbara R. Beatty

Correlation between the metabolic steps in ribosome biogenesis and nucleolar ultrastructure is being studied in amphibian oocyte nucleoli.[1] During oocyte growth, synthesis of rRNA precursor molecules (r-pre-RNA) occurs in the hundreds of extrachromosomal nucleoli in the oocyte nucleus. Each 40S r-pre-RNA molecule gives rise to one 18S and one 28S rRNA molecule. Thin-sectioned nucleoli exhibit a compact fibrous core surrounded by a granular cortex. When isolated unfixed into dilute saline, these components separate and disperse. Under conditions which completely separate nucleolar cores and cortices and allow maximum unwinding of the cores, each core is observed to consist of a thin circular axial fiber periodically coated with matrix material. DNase breaks the core axis, whereas proteases and RNase remove the matrix material.

The RNP matrix units exhibit a thin-to-thick gradation, and each unit shows similar orientation along the circular axis. Each matrix unit is separated from its neighbors by matrix-free segments of the core axis (Fig. 19.11.1). Following short term in situ labeling of RNA in intact oocytes, electron microscopic autoradiographs of unwound nucleolar cores show label over the matrix units but not over matrix-free "spacer" segments. Since this initial synthesis corresponds in time to the appearance of labeled 40S r-pre-RNA in nuclear fractions, the portions of each core axis coated with matrix must be the cistrons for the 40S r-pre-RNA molecule. The cistrons are visualized by virtue of the fact that many r-pre-RNA molecules are being transcribed simultaneously on each cistron.

Nucleolar core axes are stretched to variable degrees, depending on the preparative procedures. When core axes are uniformly stretched, the matrix units are similar in length. With minimal stretching, matrix units are 2 to 2.5 μ long. After severe stretching, matrix units slightly over 5 μ long have been

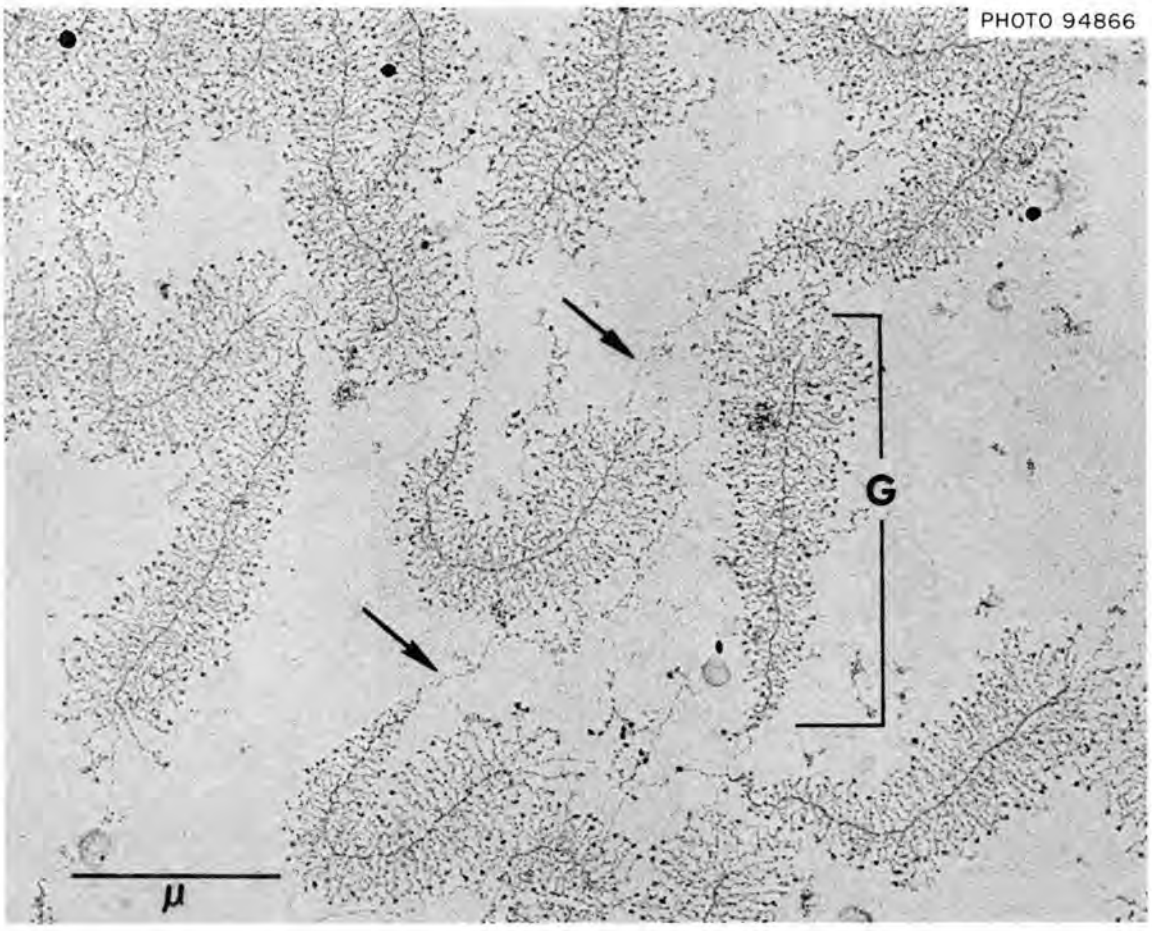

Fig. 19.11.1. Visualization of Genes.

observed. The "spacer" segments between cistrons show, in addition, differences in length independent of stretching variations. Most "spacers" are approximately one-third the length of the matrix units, but "spacers" up to ten times as long as neighboring cistrons have been observed.

In two separate studies the molecular weight of the 40S r-pre-RNA molecule in amphibians has been estimated to be 2.5×10^6 (ref. 2) and 3.5×10^6 (ref. 3). The length of unstretched r-pre-RNA cistrons corresponds closely to the length of B conformation DNA needed to code for an RNA molecule with a molecular weight 2.5×10^6, the lower of the estimates for the r-pre-RNA molecule. However, stretched matrix units slightly over 5 μ long have been observed. If the lower-molecular-weight estimate is correct, then stretching a cistron to twice its length ($2 \times 2.5\ \mu$) would require that the helical structure of the DNA be destroyed and the polynucleotide backbones extended to their maximum length. Since the DNA of the nucleolar core axes is coated with protein, such extreme elongation seems highly unlikely. A higher molecular weight than the lower estimate for the r-pre-RNA molecule would seem more in accord with the observed lengths of maximally stretched cistrons.

At the time of active r-pre-RNA synthesis in the extrachromosomal nucleoli of amphibian oocytes, each r-pre-RNA cistron is separated by a length of core axis that apparently is not transcribed. Whether these segments ever are used for genetic transcription is not known. Biochemical analyses of the rDNA satellite from both somatic[2,3] and ovarian[4] tissues of *Xenopus laevis* show that a significant portion of the nucleolus organizer DNA is not homologous to rRNA. These studies also show that the nonhomologous component of the rDNA satellite has a high GC base content and interdigitates with the cistronic stretches coding for rRNA. Since the matrix segments of the core axes

contain the DNA which is homologous to rRNA, the nontranscribed "spacers" must contain high GC nonhomologous DNA.

Although the nucleolus organizer on the chromosome itself has not been examined, every cistron so far observed in extrachromosomal nucleoli has had adjacent "spacers." Also the diameter of stretched "spacers" indicates that the continuity of each core axis is maintained by a double-helix DNA molecule. It seems highly likely that this structural arrangement exists in the chromosome locus itself. If so, the actual length of the DNA in the nucleolus organizer of the genome would be considerably underestimated by rRNA-DNA hybridization since each "spacer" is at least one-third the length of each r-pre-RNA cistron.

When the matrix units are examined in greater detail, each is observed to consist of fine individual fibrils of increasing lengths connected by one end to the core axis (Fig. 19.11.1). There are ~100 fibers per matrix unit. Each fibril presumably consists of a growing r-pre-RNA molecule coated with protein. In *Xenopus*, estimates of the redundancy of nucleolar genes in the 1C (haploid) genome range from 450 (ref. 3) to 800 (ref. 2), and estimates of the amplification of the 1C genome in the oocyte range from 1500 X 1C (ref. 5) to 5200 X 1C (ref. 4). If all the r-pre-RNA cistrons are active at the same time, as observations on rapidly growing oocytes indicate, then somewhere between 67.5×10^6 and 416×10^6 polymerases are simultaneously transcribing presumably identical r-pre-RNA molecules in a single *Xenopus* oocyte nucleus.

The number of cistrons per nucleolar core varies considerably. For example, in *Xenopus*, cores with 8, 9, 10, 15, 16, 26, 60, 65, 72, 92, and 175 cistrons have been observed. Reasonably accurate estimates of up to 1000 cistrons for larger cores can be made, but estimates on the largest cores are impossible. These estimates indicate that nucleolar cores exist which may contain either more or less cistrons than the redundancy estimated for the 1C chromosomal locus.

Extrachromosomal amplification of the nucleolar DNA occurs at the pachytene stage of oogenesis,[4] but the molecular aspects of the amplification are entirely unknown at present. However, the amplification mechanism must account for the diversity in the numbers of cistrons per core.

Future research using this system will be directed toward observing structural aspects of derepression and repression of these genes during oogenesis, and the fine structure of the sequential steps in the processing of the large r-pre-RNA molecule in the nucleolus.

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19.12 ESTROGEN-INDUCED HEPATIC SYNTHESIS OF A SERUM LIPOPHOSPHOPROTEIN AND ITS SELECTIVE UPTAKE BY THE OVARY AND TRANSFORMATION INTO YOLK PLATELET PROTEINS IN *XENOPUS LAEVIS*

R. A. Wallace D. W. Jared

In previous studies,[1] we have shown that when a gonadotropin is administered to adult female *Xenopus laevis*, they ovulate their mature oocytes and undergo a period of vitellogenesis lasting for 30 to 40 days. During this time, estrogen synthesis is enhanced, and a serum lipophosphoprotein (SLPP) with a physiological half-life of only two days appears in the circulation, and, simultaneously, many immature oocytes enlarge as a consequence of protein yolk deposition. If the vitellogenic females are ovariectomized, the physiological half-life of SLPP in the serum changes to about 40 days, thus suggesting that the maturing ovary is normally responsible for the removal of SLPP from the circulation. SLPP with a physiological half-life of 40 days also appears in the circulation of estrogen-treated males.[1,2]

Synthesis of Serum Proteins and SLPP by *Xenopus* Liver in Vitro. – The extent to which the liver is responsible for the synthesis of components found in the serum was ascertained directly by culturing liver slices in a modified Liebovitz-L 15 medium in the presence of [^{3}H]leucine. The time course for the appearance of labeled protein in the medium indicated that liver from animals under different physiological conditions continuously released protein into the medium over at least a four-day period. Livers from vitellogenic females and estrogen-treated males, however, appeared much more active in this respect than those from normal females or males. Chromatograms of the culture medium after incubation indicated that liver pieces from all the animals synthesized and released

labeled serum proteins at approximately the same rate and that the main reason for the different amounts of radioactive protein released during the time course experiments was due to the relative amount of labeled protein which eluted in the position for SLPP.

Incorporation of SLPP by the Ovary. – Estrogen-treated females synthesized and released SLPP into their blood stream, but the SLPP accumulated in the circulation and did not disappear until a gonadotropin preparation was subsequently injected. Thus, estrogen or the mere presence of SLPP does not promote an uptake of SLPP by the ovary, but rather this process is under a hormonal control mechanism separate from that regulating SLPP production.

To indicate whether or not the uptake of SLPP by the ovary is a selective process, equivalent amounts of ^{3}H-labeled SLPP and two serum proteins were injected into three groups of vitellogenic females. Time studies indicated that SLPP left the circulation and became associated with ovarian protein approximately 50 times more rapidly than either of the other two serum proteins.

Transformation of SLPP into Yolk Proteins. – In order to explore the relationship between SLPP and the yolk proteins lipovitellin and phosvitin, doubly labeled SLPP (with [^{3}H] leucine and [^{32}P] phosphate) was injected into vitellogenic females, and high-salt extracts were made of the ovaries at various times afterward and chromatographed on TEAE-cellulose. The labeling found in such extracts several hours after [^{3}H, ^{32}P]SLPP injection indicated the presence of a single component eluting in the position for SLPP and which had an ^{3}H/^{32}P ratio similar to the injected [^{3}H/^{32}P]SLPP. Eight hours after injection, the labeling pattern appeared rather heterogeneous and by twenty hours, virtually all of the ^{3}H-label eluted in the position for lipovitellin and most of the ^{32}P-label eluted in the position for phosvitin. No other cellular proteins were labeled. Since lipovitellin contains 98% of the leucine in the yolk platelet crystal and phosvitin contains 79% of the protein phosphorus, isotopic markers for these two components serve as fairly specific indicators for lipovitellin and phosvitin respectively. It appears therefore that SLPP is a complex comprised of the precursor molecules for lipovitellin and phosvitin and that once the complex is selectively incorporated by the ovary, an intramolecular rearrangement occurs whereby it is transformed into the two proteins of the yolk platelet crystal.

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19.13 ISOLATION AND CHARACTERIZATION OF THE FEMALE PROTEIN FROM THE CECROPIA MOTH

Muh-liang Pan R. A. Wallace

A sex-limited protein detected in the blood of *Hyalophora cecropia* by immunological methods is specifically sequestered by the oocyte during vitellogenesis.[1] In order to understand better the mechanisms responsible for the synthesis of this protein and its selective uptake by the oocyte, we felt it desirable to develop a simple method for the isolation of the female protein in reasonable yield as a first step and to provide some molecular characteristics of the purified product.

A Tris-citrate buffer system compatible with both blood and yolk proteins of the cecropia moth was used for chromatography on DEAE-cellulose. Gradient elution conditions were established with the femal blood whereby the female protein, representing 10% of the total protein, eluted as a single homogeneous peak after most of the other protein material flushed off the column. Chromatograms of yolk extracts indicated that 80% of the protein in the extract eluted in the same position as the female protein, whereas chromatograms of male blood established that the female protein component was completely absent.

Examination of the purified material in the ultracentrifuge by sedimentation equilibrium and velocity methods revealed a homogeneous protein with $d \log f/dr^2 = 1.495$ at 11,264 rpm and $s^0_{20,w} = 15.9$S respectively. An effective Stokes radius, determined on a calibrated 6% agarose column (effective pore radius = 30.6 mμ), was found to be 6.46 mμ, corresponding to a diffusion coefficient of 3.31×10^{-7} cm^2/sec. The partial specific volume, determined pycnometrically, was 0.765 ml/g. Thus the molecular weight of the *Hyalophora* female protein was found to be 5.0×10^5 using the classic Svedberg formula or 5.2×10^5 using the sedimentation equilibrium equation.

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19.14 YOLK-PRECURSOR-PROTEIN SYNTHESIS IN THE BLUE CRAB

Marilyn S. Kerr

The soluble protein in eggs of many decapod crustaceans consists chiefly of a high-density lipoprotein,

characterized and termed lipovitellin by Wallace *et al.*[1] A lipoprotein present in hemolymph sera of adult female blue crabs appears, by electrophoretic and immunochemical analyses, identical to lipovitellin extracted from blue crab oocytes. Electrophoretic analyses of sera from a large number of female blue crabs showed that this serum component was present in highest concentrations in females whose eggs were undergoing vitellogenesis, indicating that the lipoprotein might be synthesized external to the ovary and transported to the oocytes by the hemolymph.

In vitro culture of various tissues with ^{14}C-labeled amino acids was undertaken in an attempt to determine the site of synthesis of the serum lipoprotein. After culturing, proteins in the media were separated by paper curtain electrophoresis and reacted with immunochemical reagents specific for lipovitellin. Dissolved precipitates were assayed for radioactivity.

Results indicate that cells circulating in the hemolymph of females having maturing oocytes are capable of synthesizing the serum lipoprotein. Although the electrophoretic mobility of the lipoprotein elaborated in vitro differed slightly from that of normal serum lipoprotein, quantitative precipitin tests showed that the protein moieties bound by the specific antibody were equal. While further characterization of the in vitro synthesized and normal serum lipoproteins, as well as lipovitellin, will be requisite to understanding the mechanisms involved in vitellogenesis, it seems probable that in this crustacean, as in vertebrates, a yolk protein or its precursor can be synthesized outside the ovary.

In addition to being of interest from a developmental point of view and as an in vitro-protein-synthesizing system, the fact that hemocytes can apparently synthesize a yolk protein could provide a convenient assay (in vivo) for studying hormonal control of oogenesis in crustaceans.

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19.15 CHROMATOGRAPHIC ISOLATION OF CRUSTACEAN HEMOCYANINS

Marilyn S. Kerr

Hemocyanin (HCy), the copper-containing respiratory pigment of many Arthropoda, Mollusca, and Xiphosura, has been the subject of numerous types of investigations. Physical and chemical studies of crustacean HCy have resulted in conflicting statements from different laboratories and about HCy obtained from different species. Part of the conflict might stem from the fact that most investigators have used whole hemolymph serum, assuming that HCy is the major, if not the only, protein present. Many crustaceans, however, have proteins other than HCy in their hemolymph. To determine whether or not a chromatographic procedure suited to isolating serum proteins of the blue crab *Callinectes sapidus*[1] might be applicable to serum from other crustaceans, it was decided to investigate hemolymph from two widely divergent decapods – the Bermuda land crab, *Gecarcinus lateralis*, and the sluggish spider crab, *Libinia emarginata*.

Hemocyanins of all three species were eluted from the DEAE-cellulose columns in identical positions and, after concentration, rechromatographed in the same position. No slow-HCy, such as exists in the serum of some female blue crabs, was detected in sera from specimens of *G. lateralis* or *L. emarginata*. *C. sapidus* fast-HCy and HCy from the other two species had copper-protein ratios of about 0.2:100. That the rechromatographed hemocyanins were still active in oxygen-combining properties was shown by their blue color after oxygenation and peroxidase activity. Apohemocyanins (HCy after removal of copper) of the three species were prepared from HCy as first isolated and from rechromatographed HCy. These apoproteins chromatographed in positions identical to the native HCy.

These results indicate: (1) that HCy molecules from the three species are quite similar; (2) that removal of copper from HCy does not alter, significantly, the molecular charge; and (3) that the chromatographic procedure might be useful in isolating HCy from different crustaceans.

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19.16 ON HEMOCYTES

Marilyn S. Kerr J. N. Dumont

Further characterization of the hemocyanin-synthesizing capacity of tissues of the blue crab by in vitro culture[1] suggests that the respiratory protein is synthesized by cells "circulating" in the hemolymph. Synthetic activity originally observed in cultures of other tissues disappears if these tissues are washed carefully before incubation with ^{14}C-labeled amino acids, indicating that the cells responsible for hemocyanin synthesis must adhere to the surfaces of organs.

The fact that cells in the hemolymph or hemocytes are responsible for hemocyanin synthesis and, in vitellogenic females, for lipoprotein synthesis led to an interest in the types of cells present. Published microscopic examinations have revealed numerous (usually five) different types of cells and suggested that each type represents a stage in maturation of a simple monocyte-like cell to a large granulocyte.[2]

Preliminary investigation indicates that two of the cell types formerly described are probably based on different types of reactions of the amoebocytic cells when placed on glass slides. Autoradiography of hemocytes collected at specific time intervals after a single injection of ^{3}H-thymidine reveals that the monocyte-type cells are active in DNA synthesis. The reduction of number of grains per cell and the increase in the labeled cell population suggest that these cells are undergoing mitosis regularly. No labeled granulocytes have been observed during the 70 days following the injection of thymidine. These results support the suggestion that the granulocytes may indeed arise from the small monocyte-type cell after an extensive growth phase.

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19.17 CYTOLOGICAL STUDIES OF DEVELOPMENTAL PHENOMENA IN *XENOPUS* OOCYTES

J. N. Dumont M. Virginia Cone

Ovulation in *Xenopus* can be induced by a single injection of human chorionic gonadotrophin. Following ovulation a "vitellogenic" period occurs during which some oocytes remaining in the ovary acquire yolk and become mature. Detection of these maturing oocytes is possible after injection of Trypan Blue into the vitellogenic female. Only those oocytes actively accumulating yolk incorporate the dye. Electron microscopic examination of these maturing oocytes, which range from 580 to 1200 μ in diameter, reveals greatly enhanced micropinocytotic activity. "Coated" micropinocytotic pits develop along the walls of deep crypts which invaginate into the oocyte and increase its surface area and form spaces between microvilli. The internalized micropinocytotic vesicles contain a core of dense material, presumably the yolk precursor. Within the cortical ooplasm some of these vesicles fuse with each other and form precursor yolk platelets, while others elongate to form tubules which contain the dense yolk precursors. The tubules eventually fuse with the developing yolk platelets. As these yolk platelets continue to enlarge by the addition of yolk, their contents begin to crystallize, and they appear as small, but typical, yolk platelets. The formation and fate of micropinocytotic vesicles have been followed by the use of two electron-opaque markers: horseradish peroxidase and ferritin. Both substances are incorporated by the oocyte and are eventually deposited in the yolk platelet. They are, however, excluded from the crystalline portion and remain in the superficial layer of the platelet.

Some micropinocytosis occurs in mature oocytes and oocytes from nonvitellogenic females. However, in these cases the vesicles are small and appear to be empty. In addition, the crypts which penetrate into the cytoplasm from the surface of the vitellogenic oocyte are greatly reduced. There is no evidence for the involvement of oocyte organelles (e.g., ER, Golgi) in the synthesis of yolk proteins.

Continuing research involves techniques of fluorescence microscopy, and light- and electron-microscopic methods of autoradiography and histo- and cytochemistry to examine in detail other developmental aspects of oocyte maturation in *Xenopus*, such as: (1) the origin and composition of the superficial layer of the yolk platelet, (2) the changes in oocyte circulatory elements which can be correlated with yolk transport and uptake, (3) the possible function of the follicular epithelium in steroid synthesis, (4) the nature of the "coats" on micropinocytotic pits and their relationship to the specificity of yolk uptake, and (5) the origin and development of organelle systems throughout maturation.

20. Biochemistry of Cell Differentiation

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20.1 REGULATION OF RIBOSOMAL RNA SYNTHESIS AND RIBOSOMAL ASSEMBLY IN LENS EPITHELIAL CELLS

John Papaconstantinou Emilia M. Julku

The epithelial cells of the adult vertebrate lens are in an extended G_1 phase of the cell cycle and can remain in this stationary phase for very long periods of time without having permanently lost the ability to divide. When the intact lens is placed in organ culture, the epithelial cells will come out of the stationary phase and start to divide. Because of these interesting cell cycle characteristics, these cells can be effectively used to study the regulation of macromolecular synthesis during different phases of the cell cycle and in a prolonged stationary phase. In one of our recent publications,[1] we have shown that ribosomal RNA synthesis as well as the processing of rRNA into the ribosome are regulated during the stationary phase. The transcriptional regulation of rRNA synthesis may be seen when, as a result of organ culturing, we found a tenfold increase in the rate of rRNA synthesis. No further work has been done to further understand the repression of rRNA synthesis during the stationary phase and its derepression when the cells divide.

With respect to the processing of rRNA during the formation of the ribosome, we have reported[1] that the 45s precursor is synthesized and the 32s RNA is formed. In the stationary phase, however, these precursor RNA molecules are not completely chased into the 28s and 18s rRNA. Similar pulse-chase experiments with dividing epithelial cells show that the precursor material is chased into the 28s and 18s forms. Thus there appears to be a regulation of the processing of precursor rRNA during the stationary phase.

In our more recent work we have been able to isolate precursor rRNA and 28s and 18s rRNA by acrylamide gel electrophoresis and have confirmed the above observations by this procedure. Furthermore, we have also shown by acrylamide gel electrophoresis the existence of a 22s and 17s rRNA species in the stationary cells. The 22s rRNA may be either a breakdown product of 28s rRNA or a precursor to 18s rRNA; the 17s may be a breakdown product of either 28s or 18s rRNA. The relationship of these rRNA species to 28s and 18s rRNA will be determined by DNA-RNA hybridization techniques.

Finally, we propose to concentrate during the next year on the transcriptional regulation of rRNA synthesis in these cells. Experiments are now being carried out to determine whether the tenfold increase in rRNA synthesis is due to an initial amplification of the rRNA cistrons or whether there is a derepression of an existing nonamplified ribosomal genome.

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20.2 THE REGULATION OF ERYTHROPOIESIS

W. S. Bradshaw John Papaconstantinou

Recent data demonstrate that as much as 40% of the DNA from a wide variety of animals is present as repeated nucleotide sequences.[1] In the amphibian oocyte the genes coding for ribosomal RNA have been shown to be repeated many times. Furthermore, at a specific time during the development of the oocyte, the ribosomal cistrons have been shown to amplify many times, to account for the increased amount of rRNA needed for ribosome formation.[2] Amplification of a specific region of the genome has only been shown to occur for the ribosomal cistron. Experiments are being carried out to determine whether such amplification can occur during cellular differentiation and especially at a time when the initiation of tissue-specific protein synthesis occurs. We have chosen to study the synthesis of hemoglobin by erythropoietic tissues to determine whether specific gene amplification can occur during cellular differentiation. In these studies severe anemia is induced in mice by successive subcutaneous injections of phenylhydrazine. It was demonstrated histologically that 50 to 80% of the red cells in the circulating blood are nucleated erythroblasts after three to five days of phenylhydrazine treatment. ^{3}H-thymidine was also administered during the latter part of the injection series. DNA was then prepared[3] from red blood cells and from liver and spleen nuclei and was banded by equilibrium density gradient centrifugation on CsCl. The labeled DNA from nucleated RBC and liver shows a single peak, with a shoulder on the light side of the main band. The profile from the spleen DNA also shows the light satellite DNA. However, there are indications of the presence of other populations of DNA on the light side of the main band which are not associated with the light satellite. Experiments are now in progress to rule out the possibility of artifacts and to further characterize the DNA from the spleen.

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20.3 ESTROGEN-INDUCED GENE AMPLIFICATION IN THE MALE *XENOPUS LAEVIS*

J. M. Collins John Papaconstantinou

Molecular hybridization experiments have revealed that the genes for ribosomal RNA (rRNA) are differentially replicated in oocyte of *Xenopus laevis.*[1] This amplification of DNA apparently enables the oocyte to meet the increased demands for rRNA made upon it at a particular stage in its life cycle. Wallace and Jared[2] have demonstrated that the hormone 17B estradiol stimulates the liver of male *Xenopus* into synthesizing a serum lipophosphoprotein which is characteristic of vitellogenic females. Furthermore, electron microscopic studies show a proliferation of liver ribosomes in estrogen-treated males.[3] We propose to determine if gene amplification might be one mechanism whereby the increased synthesis of a structural protein could be accomplished.

We have found that the injection of 0.1 mg of estrogen leads to an increase in the amount of ^{3}H-thymidine incorporated into nuclear DNA. The increase is seen 4 hr after estrogen treatment. When this nuclear DNA is banded in a cesium chloride gradient, all the radioactivity appears to be somatic DNA. No label has been detected in the heavy region which would correspond to ribosomal DNA. Experiments are in progress to measure the amount of ribosomal DNA synthesized by hybridization with rRNA.

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20.4 DOES AN ACETYLATED SPECIES OF AMINOACYL tRNA EXIST IN BOVINE LENS?

J. M. Collins John Papaconstantinou

The formylation of methionyl-tRNA has been shown to be the controlling step in the initiation of protein synthesis in bacteria.[1] The *N*-terminal amino acid of lens α-crystallin is *N*-acetylmethionine.[2] The possibility was thus raised that *N*-acetylmethionyl-tRNA might exist in lens cells. We searched for this RNA by exposing intact lenses that had been in organ culture to ^{3}H-acetate along with a mixture of amino acids and fractionating the tRNA on a MAK column. No acetylated aminoacyl tRNA could be detected.

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20.5 MECHANISM OF RIBOSOMAL ASSEMBLY DURING LENS CELL DIFFERENTIATION

W. A. Elmer John Papaconstantinou

Studies are being carried out to determine if the assembly of lens ribosomal RNA and protein during ribosome formation is regulated during lens cell differentiation and during stages of the cell cycle in which the cells are stationary. We are concerned with the following questions: (1) Are there qualitative or quantitative differences in the ribosomal proteins associated with ribosomes in the epithelial cell and fiber cell? (2) Is there a selective synthesis of the ribosomal proteins when the epithelial cell is in an extended G_1 stationary phase or when the epithelial cell has differentiated into a fiber cell? (3) What is the relationship between the size of the ribosomal protein pool and the assembly of the complete functional ribosome?

Ribosomal proteins have been extracted and resolved by acrylamide gel electrophoresis. No qualitative differences were found between epithelial cell and fiber cell ribosomal proteins. Examination of the proteins isolated from 60s and 40s ribosomal subunits indicates that there are both qualitative and quantitative differences between the proteins of these subunits.

Experiments were carried out to study ribosomal protein synthesis and to determine whether there are differences in the rate of synthesis of these proteins. Lenses were incubated in the presence of [^{14}C] amino acid mixture for 3 hr, and ribosomal proteins were prepared from the epithelial and fiber cells. In one series of experiments adult lenses were used. The epithelial cells from these lenses are in an extended G_1 phase of the cell cycle, whereas the fiber cells are in a permanent stationary phase. These experiments showed that epithelial cells in the extended G_1 stationary phase incorporate [^{14}C] amino acids into ribosomal proteins but at a very low rate. The overall rate of protein synthesis in the fiber cell was found to be quite low, and there was essentially no detectable incorporation of amino acids into the ribosomal proteins. These data indicate that cells in the stationary phase show a low rate of turnover of ribosomal proteins.

As a result of these observations, experiments were carried out to determine the extent of the ribosomal protein pool in these cells. Lenses were preincubated for 3 hr in the presence of [^{14}C] amino acids, and at the end of the incubation period protein synthesis was inhibited with cycloheximide. This permitted a labeling of the ribosomal proteins in the pool, and upon inhibition of protein synthesis the labeled ribosomal proteins in the pool could be followed by their incorporation into the ribosome. The data showed that there was, in the epithelial cells, a progressive incorporation of radioactive ribosomal proteins into the ribosomes after the addition of cycloheximide. Thus there is a detectable ribosomal protein pool (and a turnover of ribosomal proteins) in these stationary epithelial cells. On the other hand, cycloheximide treatment did not result in a similar progressive increase in the specific activity of the ribosomal proteins in the fiber cell ribosomes. We conclude from these observations that there is a significant ribosomal protein pool in the epithelial cells which does not exist in the fiber cells.

To determine whether cycloheximide affects RNA synthesis, [^{3}H] uridine was added simultaneously with the inhibitor. A time course analysis showed that there was a tenfold stimulation of RNA synthesis as a result of cycloheximide treatment. When the 80s ribosomes were dissociated into their 60s and 40s subunits, most of the radioactive RNA peaked in the 45s region. An analysis of the RNA extracted from these ribosomal preparations showed that the radioactive RNA was dispersed throughout a 10 to 30% sucrose gradient. Preliminary evidence indicates that this RNA is nonribosomal. This stimulation of RNA synthesis by an inhibitor of protein synthesis was not observed to occur in the same lens cells when they are stimulated to reenter the cell cycle. We interpret these observations to indicate, therefore, that a (protein) repressor is synthesized during the stationary phase and that the stimulation of RNA synthesis is due to inhibition of synthesis of this repressor by cycloheximide.

20.6 REGULATION OF α-CRYSTALLIN SYNTHESIS

Winifred G. Palmer John Papaconstantinou

α-Crystallin is a large, complex protein which can be fractionated by DEAE-cellulose chromatography into seven subunits. Embryonic bovine α-crystallin is a relatively simple protein composed of two major types of subunits (subunits 1 and 4) and several minor subunits. A third major subunit (subunit 5) appears in α-crystallin after birth, while the adult protein consists of four major subunits (1, 4, 5, and 7). This successive appearance of different subunits at different stages of growth suggests that the synthesis of these different polypeptide chains is independently regulated. If this is the case, then it is likely that they are synthesized from independent mRNA's rather than from a polycistronic message. If they are synthesized from a polycistronic mRNA, then the rates of synthesis of the subunits should be inhibited to the same degree by actinomycin. However, if the subunits are synthesized from individual mRNA's with varying half-lives, then one should observe a differential effect of actinomycin upon the rates of subunit synthesis. To test this, adult lenses were incubated with ^{14}C-amino acids in the presence and absence of actinomycin for 4 hr. Epithelial cells were separated from fiber cells, and the α-crystallins were extracted and fractionated into their subunits.

Actinomycin causes a striking differential effect upon the synthesis of α-crystallin subunits in the epithelial cell. The rate of synthesis of subunit 7 is inhibited by 43%, whereas subunit 1 is only inhibited by 7%. The incorporation of ^{14}C-amino acids into subunits 4 and 5 is stimulated by actinomycin.

A differential effect upon the synthesis of α-crystallin subunits in fiber cells was also observed. The synthesis of all of the subunits was stimulated, but this stimulation ranged from 5% for subunit 7 to 50% for subunit 4. These data suggest that the individual α-crystallin subunits are synthesized from individual mRNA's of varying stabilities.

One problem which has hampered the interpretation of these data is that the chromatographic resolution between subunits 3 through 6 has been insufficient. That there is a considerable overlap of proteins between the different subunit peaks has become evident with acrylamide gel electrophoresis. An attempt has been made to design a new column elution system to improve the resolution of the subunits. It was found that the addition of mercaptoethanol to the initial α-crystallin preparation and to the buffers used in the DEAE-cellulose chromatography substantially altered the elution patterns of the α-crystallin subunits. The protein elutes as four distinct peaks; the minor subunit bands are no longer present. The electrophoretic patterns on acrylamide gels indicate that each subunit peak now contains unique protein fractions. The actinomycin experiments were repeated with this new elution system, and the earlier results were confirmed.

21. Enzymology

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Protein Synthesis (21.1–21.10)

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Enzymatic Mechanisms in Protein Synthesis (21.11–21.14)

M. P. Stulberg[a]
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J. G. Farrelly[a,h]
K. R. Isham
Barbara H. Chastain

Mammalian Cell Regulation (21.15–21.20)

K. Bruce Jacobson[a]
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Participation in Cooperative Programs:

AEC-NIGMS (30.51, 30.52)
AEC-NCI Cocarcinogenesis (30.3–30.8)
AEC-NCI-NIGMS (30.53)

[a]Dual Assignments
[b]Visiting Investigator from Abroad
[c]SRC/NATO Postdoctoral Fellow
[d]USPHS Senior Postdoctoral Fellowship
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21.1 DIFFERENCES OF ISOACCEPTING VALYL-tRNA's IN THE RETICULOCYTES OF C3H AND C57BL MICE

K. M. Hilse[1] Wen-Kuang Yang

It has been proposed that modifications in the tRNA of the cell may play an important role in the cellular regulatory mechanism. Since it has been shown in a separate study[2] that the C57BL mouse has only one kind of hemoglobin α-chain whereas the C3H mouse has an additional kind of α-chain which amounts to 70% of the total α-chains in the hemoglobin, research was initiated to examine what role the tRNA's may play in the synthesis of the mouse hemoglobin.

In a pilot study it was established that upon incubation with radioactive amino acids the reticulocytes from the phenylhydrazine-treated C3H mice synthesized the two kind of α-chains in the same 70/30 ratio. tRNA and aminoacyl-tRNA synthetases were prepared from the reticulocytes of C3H and C57BL mice respectively. Aminoacylation of C3H reticulocyte tRNA with the homologous enzyme showed no competition between the two amino acids occupying the same position within the α-chain (gly/val; val/ile; asn/ser), indicating that no tRNA was recognized ambiguously by the synthetases of C3H mouse reticulocytes. Comparison of various isoaccepting aminoacyl-tRNA's from the C3H and C57BL mouse reticulocytes was performed on the

Freon reversed-phase column (RPC-2) by the method described.[3] The reticulocytes from both strains of mice showed similar to identical patterns of isoleucyl-, seryl-, glycyl-, methionyl-, and leucyl-tRNA's. Interestingly, valyl-tRNA's from the reticulocytes of the two strains of mice revealed marked differences in their chromatographic patterns. Three valyl-tRNA peaks were observed in the preparation from C57BL reticulocytes; C3H reticulocyte val-tRNA's yielded four to five peaks, with one additional prominent peak.

Further characterization of these differences in valyl-tRNA's demonstrated: (1) tRNA isolated from a mixed sample of C3H and C57BL reticulocytes gave an intermediate pattern of valyl-tRNA's on RPC-2; (2) the characteristic profiles of valyl-tRNA's in the two strains of mice persisted when the labeled valyl-tRNA was prepared either in the presence or in the absence of 19 other cold amino acids; (3) the radioactivity carried by each peak was identified as valine by descending paper chromatography, after alkaline discharge from tRNA; (4) liver from the two strains of mice produced the same differences in valyl-tRNA's as the reticulocytes did.

In summary, tRNA's from reticulocytes synthesizing one or two kinds of hemoglobin α-chains are precisely recognized by their homologous specific synthetases. Since among various tRNA's specific for the amino acids involved in the duplex positions of α-chain in C3H mouse, only valyl-tRNA's showed different patterns, it is suggested that the additional isoaccepting species of valyl-tRNA may be required for the translation of the additional α-chain in C3H mouse rather than serve to modify the codon recognition.

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21.2 POSSIBLE ROLE OF tRNA IN THE REGULATION OF MOUSE HEMOGLOBIN ALPHA CHAIN SYNTHESIS: AN IN VITRO DEMONSTRATION

Wen-Kuang Yang R. A. Popp[1]
K. M. Hilse[1] G. David Novelli

Based upon the observations that the C3H strain of mouse has two kinds and the C57BL strain of mouse has only one kind of hemoglobin alpha chain in red blood cells[2] and that these two strains of mice showed different chromatographic patterns of valyl-tRNA's in the reticulocytes,[3] experiments have been carried out to study the mechanism of mouse hemoglobin synthesis in vitro and to examine in what manner the isoaccepting tRNA's may be related to the synthesis of hemoglobin. An efficient and more defined cell-free system of amino acid incorporation has been designed with the object that peptide synthesis may be dependent on the exogenously added tRNA and that the system may be able to initiate new chains in addition to nascent peptide completion. Polyribosomes were isolated from reticulocytes by a discontinuous sucrose gradient centrifugation. By employing DEAE-cellulose column chromatography, a tRNA-free enzyme preparation containing all synthetases and transfer factors was made from the reticulocyte 198,000 $\times$ *g* supernatant. With polyribosomes, the enzyme preparation, and an ATP-generating system, tRNA was found to stimulate amino acid incorporation. After 1 hr of incubation 50 to 60% of the radioactive amino acid appeared in the released peptide chains. Sephadex G-25 gel filtration revealed that the released peptides were excluded from the column accompanying the carrier hemoglobin. This in vitro system of peptide synthesis was performed with various combinations of ribosomes and tRNA from C3H and C57BL mouse reticulocytes. The released hemoglobin and the nascent chains were digested with trypsin, and the tryptic peptides were then isolated by high-voltage electrophoresis and paper chromatography. Analysis of the labeled peptides revealed that the C57BL reticulocyte ribosomes synthesized one alpha chain either with its homologous tRNA or with C3H reticulocyte tRNA, indicating that they contain only one type of alpha chain messenger. When C3H reticulocyte ribosomes were incubated with its homologous tRNA, two types of alpha chain were formed in a 70/30 ratio, as determined by measuring the quantity of labeled 25-(Val)αT4 and 25-(gly)αT4. With C3H ribosomes and C57BL tRNA, the synthesis of 25-(Val)αT4 was slightly reduced. The magnitude of this reduction, however, was relatively small and suggested that the C57BL reticulocyte tRNA might modify the translation of alpha chain messages on the C3H ribosomes at a site proximal to α25 position. Experiments are in progress to determine the kinetics of amino acid incorporation into various peptides, which may help to locate exactly the site of regulation in the translation of the genetic messages for the C3H alpha chains.

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21.3 COMPARISON OF tRNA FROM SPLEENS OF YOUNG AND OLD RATS

C. J. Wust D. G. Ridner G. David Novelli

In the aging mouse, Makinodan and Peterson[1] have shown that there is a steady decline in immunological capacity resulting from the depletion of progenitor cells capable of producing antibody-synthesizing progeny. It has been reported as well that "toxic factors" increase in the spleens of mice with age.[2]

The possible regulation of protein synthesis by certain species of tRNA suggested to us that an aging process may involve the synthesis and accumulation of aberrant forms of tRNA that restrict the flow of some amino acids into peptides. These ineffective species of tRNA would result from transcriptional errors or the formation of macromolecular complexes that would inactivate or repress the normal amino acid acceptor activity. Transcriptional errors could arise from the accumulation of mistakes made in rapidly proliferating cells such as are found in the spleen. They could also arise by factors that interfere with macromolecular synthesis. It might be postulated, for example, that nucleic acids under certain conditions are antigenic and that in the aging mammal, immunologically cross-reacting antibody is made that affects an autoimmune-like disease at the cellular level. This kind of immunity would be toxic by its nature.

Our first attempts to examine this problem involved a comparison of isoaccepting species of tRNA from the spleens of rats that were two or nine months of age. Spleens were homogenized and extracted with phenol at 2°C for at least 1 hr. Following the precipitation of the aqueous phase with ethanol, the precipitate was collected and dissolved in buffered medium. Ribosomal RNA was removed by adding sodium chloride to a final concentration of 1 *M*, and the soluble nucleic acids were chromatographed on DEAE. tRNA was eluted at 0.45 *M* NaCl concentration. In separate homogenates of spleens, animoacylating synthetases were fractionated from the 165,000 *g* supernatant fluids by DEAE chromatography.

Aminoacylation and formation of aminoacylated tRNA were compared using material from the two age groups and ten amino acids. There were no significant differences between the synthetases when each was used with tRNA of the same or different age group as measured in terms of millimicromoles of amino acid accepted per milligram of tRNA. The tRNA from spleens of nine-month-old animals accepted only 50% of the amount of an amino acid per $A_{260\ m\mu}$ as did the tRNA from two-month-old animals with the exception of arginine and lysine, which were 75% or greater. The fact that acceptor activity of tRNA for arginine and lysine was almost comparable suggests that there is no random inactivation or degradation of tRNA if these are the reasons responsible for the differences observed. The differences do not appear to be attributable to degraded –C–C–A terminus, since the addition of CTP and repair enzyme from *Escherichia coli* does not appreciably affect the results.

The use of the Freon reversed-phase chromatographic column has indicated some quantitative differences among the isoaccepting species of tRNA from the two age groups, but as yet no qualitative ones have been found.

Other parameters being investigated between the two age groups include in vivo methylation and thiolation of nucleotides of tRNA.

References

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21.4 COMPARISON OF TRANSFER RNA (tRNA) ISOLATED FROM SPLEENS OF RATS AND MICE

C. J. Wust D. G. Ridner

Transfer RNA (tRNA) isolated from spleens of rats or mice will accept amino acids from synthetases isolated from either species of animals. The heterologous rat synthetase–mouse tRNA combination appears to result in greater efficiency than the mouse-mouse system. We have initiated studies to examine and compare acceptor activity of tRNA isolated from either species after immunization with a common antigen, namely, sheep erythrocytes. Since the synthetases and tRNA from the two species will cross react, it is possible that the immune response to a common antigen may indicate what role, if any, tRNA plays in antibody specificity. We reported last year[1] that a comparison of acceptor activity of tRNA from spleens of nonimmunized and

immunized rats showed some quantitative differences after analyses of material by reversed-phase chromatography.

In more recent experiments, we found that after four days of immunization tRNA will accept two to three times more amino acid per A_{260} than tRNA from spleens of nonimmunized animals. The differences should not be due to a more active degradation of tRNA from nonimmunized animals during preparation, since all preparations are made similarly in the presence of ribonuclease inhibitors, unless the nonimmunized animals contain higher levels of nucleases than immunized animals or immunization results in the synthesis of nuclease inhibitors. These possibilities are being investigated.

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21.5 STUDIES ON TRANSFER RNA AND PROTEIN SYNTHESIS DURING DEVELOPMENT OF THE BRINE SHRIMP *ARTEMIA SALINA*

J. C. Bagshaw[1] F. J. Finamore[2] G. David Novelli

During the past year we have extended our study of the tRNA's of brine shrimp to include 18 amino acids. tRNA is prepared from encysted blastulae and from larval nauplii. The two types of tRNA are then used to prepare ^{14}C- and ^{3}H-aminoacyl-tRNA's, which are fractionated by reversed-phase chromatography. In comparing the chromatographic profiles of the aminoacyl-tRNA's from two stages, we are searching for changes, either quantitative (alteration of the relative proportion of isoaccepting species) or qualitative (appearance of a new species), during development.

To date, we have found quantitative changes in alanyl-, arginyl-, glutamyl-, glutaminyl-, glycyl-, isoleucyl-, leucyl-, lysyl-, and methionyl-tRNA's; aspartyl-, asparaginyl-, histidyl-, phenylalanyl-, prolyl-, seryl-, threonyl-, tyrosyl-, and valyl-tRNA's from cysts and nauplii show no differences. With only cysteinyl- and tryptophanyl-tRNA's remaining, we have not yet found a qualitative change in tRNA.

Recently we have turned our attention to an in vitro protein synthesis system derived from *Artemia*. Microsomes prepared by centrifugation at 105,000 × *g* for 90 min are very active in incorporation of ^{14}C-leucine into protein. The level of incorporation is directly proportional to the amount of microsome preparation added to an assay mixture, and under optimum conditions the system incorporates over 800 micromicromoles of leucine per milligram of ribosomal RNA. Amino acids can also be incorporated from aminoacyl-tRNA added to the assay mixture; leucyl-, methionyl-, and seryl-tRNA have been used for this purpose. We intend to fractionate some aminoacyl-tRNA's into the isoaccepting species and test each species individually for its ability to transfer the amino acid into protein in the in vitro system. We wish to use the system essentially as an assay for the presence, in endogenous messenger RNA of different developmental stages, of the codons to which individual isoaccepting species of tRNA respond.

References

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[2] Developmental Biochemistry section.

21.6 INITIATION OF RNA CHAINS BY DNA-DEPENDENT RNA POLYMERASE

Audrey L. Stevens

Whether the purified RNA polymerase interacts with native DNA in vitro at the sites that RNA chains are initiated in vivo remains unclear. The formation, in vitro, of RNA chains starting predominantly with the principal *N*-formylmethionine codon, AUG, would suggest a specific interaction. We have started a study of the 5′-terminal nucleotide sequences of RNA made with *E. coli* RNA polymerase. With T7 DNA as a template, nearest-neighbor analyses show that the 5′-terminal dinucleotide sequences of the product RNA are as follows: pppApC, 40%; pppApU, 20%; pppGpA, 16%; pppGpU, 10%; pppGpC, 10%; others, less than 5%. By T_1 ribonuclease digestion of γ-^{32}P-ATP-labeled RNA chains, it has been found that 30% start with the sequence pppAp (Up or Cp)G. The remaining 70% start with the sequence pppAp (Cp or Up) (Cp or Up or Ap)G. Examination of the pppAp (Cp or Up)G oligonucleotide is in progress. It is apparent that most of the RNA chains do *not* start with the codon AUG. It is of considerable interest, however, that most of the chains start with dinucleotide sequences which are contained in codons for amino acids other than methionine found in NH_2-terminal position in *E. coli* protein. Varying ionic and divalent cation conditions will be tried to see if they affect the terminal AUG content of the RNA chains.

Investigations of the binary complex of *E. coli* RNA polymerase and native DNA have been continued.

Results of others had shown that the binary complex, very sensitive to ionic strength, was partially stabilized to high ionic strength by incubation with only ATP or GTP. The results suggested that formation of the first phosphodiester bond resulted in a different type of binding of the enzyme to the DNA and/or oligonucleotide. In our studies with T7 DNA, we find that preincubation with single ribonucleoside triphosphates affords no stabilization of the binary complex to high ionic strength. Of the possible combinations of two, only ATP + CTP affords significant stabilization (20%). All the mixtures of three stabilize the complex significantly (12–90%). The results suggest that at least a trinucleotide must be formed before the enzyme is bound stable to ionic strength. Further investigations on whether the ionic-strength-stable bond involves the DNA or growing RNA chain are in progress.

21.7 CELLULAR DIFFERENTIATION OF THE GILL DURING SALT SECRETION

F. P. Conte L. C. Waters

Formation of enzymatic proteins, such as the Na-K-activated ATPases, appears to be a prerequisite for the gills of euryhaline fish to act as salt-secreting organs.[1] In addition, immunochemical evidence has revealed that other types of proteins are involved in the development of the electrolyte transport system.[2] Therefore the differentiating gill epithelium may be an excellent model system for the study of the active ion transport that is dependent upon the control and regulation of proteins. Whether this system is controlled at the level of transcription and/or translation is unresolved. Recent technology, particularly the chromatographic methods for analyzing individual species of tRNA, now enables one to critically examine questions concerning control mechanisms acting at the translation level.

The prime objective of the present study is to determine whether gross differences, either quantitative or qualitative, exist in the tRNA and/or the aminoacyl-tRNA synthetases present in cells actively secreting salt as compared with inactive cells. To date we have developed adequate methods for the preparation of active tRNA from both salt and freshwater gills. A synthetase preparation from freshwater tissue contains activities for all those amino acids tested (e.g., phenylalanine, aspartic acid, valine, arginine and leucine) except glutamic acid. The preparation appears to be relatively free of nuclease activity. Preliminary chromatography, on RPC-2,[3] of phenylalanyl-tRNA from gill tissue showed two activity peaks. Having worked out the essential methodology, we are now preceeding with the comparative study.

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21.8 STUDIES ON L-ASPARAGINASE AND L-SERINE DEHYDRATASE

Helen Sellin

Our attention has been directed toward two enzymes with potential use in specific therapy of nonessential amino-acid-dependent malignancies. Initially we worked with L-asparaginase, finding that enzyme activity levels varied with growth conditions and strain of *E. coli.*

At present we are interested in L-serine dehydratase, since James Regan has serine-dependent granulocytic leukemias in tissue culture. The reported serine K_m's for L-serine dehydratase of *E. coli* and *Neurospora* are approximately 3 to 5 $\times$ 10^{-3} *M.* Since the serine blood level in man is 10^{-4} *M*, an enzyme is required with a lower K_m than those known if the enzyme is to be effective in lowering the blood serine.

Using a medium of salts, glycerol as carbon source, and serine as nitrogen source, we are attempting the isolation from various soils of a microorganism with a L-serine dehydratase whose K_m for serine is less than that of *E. coli* or *Neurospora.* The assay is either ammonia nesslerization or spectrophotometric measurement of NADH depletion when L-serine dehydratase is coupled with lactic dehydrogenase and NADH. The best we have obtained so far have K_m's of the same order of magnitude as *E. coli* and *Neurospora.* We are particularly interested in an enzyme without a pyridoxal phosphate requirement, since the pyridoxal-serine Schiff base dissociation is of the order of 10^{-2} *M* and may therefore be rate-determining.

21.9 NICOTINAMIDE ADENOSINE DIPHOSPHATE GLYCOHYDROLASE

C. J. Wust Patricia M. Bihl G. J. Stine

The relationship of the amount of the enzyme nicotinamide adenosine diphosphate glycohydrolase (NADase) in the asexual cycle of *Neurospora crassa*

demonstrates that apparent increased synthesis is correlated with aging and dormancy. This relationship suggests that the synthesis of this enzyme might be a factor in senescence and aging in other living systems, including mammals. Since the enzyme would regulate the NAD-to-NADH ratio, its induction might also be important in abnormal cell differentiation and proliferation. The enzyme is specific for oxidized NAD+ and inhibited by nicotinamide, one of the products of hydrolysis.

Initially, we have developed an isolation procedure for NADase from conidia of *N. crassa.* The enzyme is prepared in high yields with high specific activity, in contrast to those preparations usually made from mycelia. The enzyme is relatively stable and is inhibited only by high concentrations of nicotinamide, whereas the mammalian enzyme is sensitive to very low concentrations. Preliminary to long-term studies in aging mice, we are examining the purification procedures for the enzyme and its kinetics, labeling it with ^{14}C-amino acids and evaluating its toxicity and half-life when administered in vivo in mice. Normally, in mice, there is a high level of enzymic activity in spleens and a lower level in the liver. The relative amounts of the enzyme have been shown to increase with age. We have also observed that during immunization of mice to sheep erythrocytes, the amount of NADase in spleen does not vary. The immune response, however, does appear to be enhanced when *N. crassa* enzyme is given as adjuvant with the antigen.

21.10 CONFORMATIONAL STUDIES ON ADH FROM *DROSOPHILA MELANOGASTER*

Peter Pfuderer K. Bruce Jacobson

The advantage in studying conformational changes in an enzyme from a genetically well-defined and well-studied species with a nice short life-span is that correlations can ultimately be made between changes in the conformation and changes in the phenotypic expression of various traits.

Alcohol dehydrogenase (ADH) exists in *Drosophila* as five isozymes, ADH_1 to ADH_5. ADH_5 is readily converted to ADH_1 in the presence of NAD_1 and can be converted back by passage through DEAE columns.

The molecular weight of ADH_5 was observed to be 60,500 by equilibrium ultracentrifugation. A partial specific volume of 0.754 was calculated from the amino acid composition data, and this also agreed with that estimated from equilibrium distribution in normal and heavy water. ADH_5 and ADH_1 were both observed to have identical sedimentation coefficients, 3.85 in water at infinite dilution. Similarly, equimolar mixtures of ADH_5 and NAD sedimented identically to ADH_5 alone. Therefore ADH_5 and ADH_1 are almost identical in size and shape, and the interconversion does not involve these parameters.

When attempts were made to try to dissociate ADH_5 into smaller subunits, a remarkable effect was observed. Reagents which did result in opening the molecule up produced a time-dependent reaggregation which ultimately resulted in complete precipitation of the sample. This reaggregation increased with temperature, hinting of hydrophobic-like bands involved in the aggregation. In the case of the pH extremes, 3 and 12, this aggregation could be stopped at a dimer of 5.6S. In urea, by keeping the solution cold, a half-molecule with a molecular weight of 31,000 was observed, but again these data were confused by the reaggregation which was also occurring. Treatment with maleic anhydride to amidate the amino groups finally resulted in a stable monomer, with enough charge to stop the aggregation.

The extrapolated value of the molecular weight of this subunit at zero centrifugal speed and low salt concentration was 15,000, which agrees with Sephadex chromatography estimates of its size. This molecular weight was observed to decrease with higher salt concentrations and centrifuge speed. The best present explanation of this phenomenon is that a lipid-like moiety was released from the molecule on dissociation, and this tended to float at increasing speed and solvent density.

The present picture of ADH_1 and ADH_5 is that of two virtually identical molecules in size and shape, both composed of four subunits of 15,000 molecular weight, but differing in the amount or attachment of NAD and in the conformation around a tryptophane residue. This tetramer may be held together by predominantly hydrophobic linkages involving a lipid moiety which has not yet been identified.

21.11 ISOLATION OF AMINOACYL-RNA SYNTHETASE: tRNA COMPLEXES

J. G. Farrelly J. W. Longworth[1] M. P. Stulberg

Introduction. – We have been continuing our studies[2] on the optical properties of the interaction of phenylalanyl-tRNA synthetase and $tRNA^{Phe}$. Although positive results have been obtained concerning fluorescence-quenching phenomena and conformational changes during the interaction, these results have not been characterized due to difficulty encountered in repeating the experiments. Therefore we have turned

our efforts toward isolation of enzyme-tRNA complexes in order to study their optical properties in a more defined and stable system.

Results and Discussion. – We have been interacting tRNA and enzyme under a variety of conditions prior to chromatography on Sephadex G-100 for the isolation of the anticipated complex. Thus far we have not been successful in demonstrating complex formation, although both enzyme and tRNA are clearly resolved during the chromatography.

Although there has been only one reported case of the isolation of synthetase-tRNA complex, the extremely small K_m's for tRNA predict that these complexes should be observed if optimal conditions are realized. We are presently testing further conditions of pH, temperature, and acceptor form of the enzyme and tRNA in order to accomplish this goal.

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21.12 PHENYLALANYL-tRNA SYNTHETASE RECOGNITION SITE IN PHENYLALANINE tRNA

L. R. Shugart M. P. Stulberg

Treatment of purified $tRNA^{Phe}$ with sodium borohydride for 3.5 hr results in the complete loss of aminoacylation activity. Base analysis shows that most of the ^3H incorporation into the tRNA from $[^3H]NaBH_4$ occurs in the 5,6-dihydrouridine and the 4-thiouridine moieties. Similarly treated $tRNA^{Val}$ and $tRNA_f^{Met}$ show only a 20% loss in activity. Kinetic studies indicate a correlation of loss of activity with ^3H incorporation into $tRNA^{Phe}$. Since two 5,6-dihydrouridine and one 4-thiouridine occur in the loop proximal to the 5′ terminus of *E. coli* $tRNA^{Phe}$, it is concluded from our results that the enzyme recognition site resides in this area and is composed of a unique base sequence and/or tertiary structure determined by this sequence.

21.13 THE STATUS OF $tRNA^{His}$ DURING REPRESSION AND DEREPRESSION OF THE HISTIDINE BIOSYNTHETIC ENZYMES IN *BACILLUS SUBTILIS*

M. P. Stulberg K. R. Isham Audrey L. Stevens

Introduction. – We have continued our efforts to demonstrate the appearance of a unique $tRNA^{His}$ in response to histidine repression or derepression.[1] In order to approximate the status of His-tRNA within the cell during different states of control of histidine biosynthesis, we have turned to pulse labeling and extraction of His-tRNA. The procedures used thus can be likened to an in vivo analysis.

Results and Conclusions. – *Bacillus subtilis* 30 contains two isoaccepting species of $tRNA^{His}$ that are relatively labile to hydrolysis when aminoacylated. Thus RPC-2 chromatography is operated at 4° and pH 4.0 to minimize deacylation of His-tRNA. With these and other modifications of procedure, there is less than 10% hydrolysis in the course of an experiment. The two species of $His\text{-}tRNA^{His}$, when studied under conditions of repression and derepression of the histidine biosynthetic enzymes, are equivalent qualitatively and quantitatively. However, derepressed cells have an average of 66% of the His-tRNA present in repressed cells in vivo. This difference is not due to the total amount of $tRNA^{His}$ present in the two states but must reflect differences in the regulation of the aminoacylation reaction in vivo.

Reference

[1] M. P. Stulberg, K. R. Isham, and A. L. Stevens, *Biol. Div. Ann. Progr. Rept. Dec. 31, 1967,* ORNL-4240, p. 256.

21.14 THE ENZYME RECOGNITION SITE IN $tRNA^{Phe}$

M. P. Stulberg K. R. Isham

Introduction. – We have previously[1] determined the locus of the enzyme recognition site in *E. coli* B $tRNA^{Phe}$. This was accomplished by the isolation of limit-digest residues of $tRNA^{Phe}$ produced by the action of highly purified venom phosphodiesterase. The digestion proceeds from the 3′ terminus of tRNA. In order to further characterize the site, we are attempting to obtain limit-digest residues of $tRNA^{Phe}$ by digestion from the 5′ terminus. This should be accomplished by the use of spleen phosphodiesterase, which is specific for hydrolysis of RNA from the 5′ end.

Results and Discussion. – The recently reported procedure[2] for isolating endonuclease-free spleen phosphodiesterase has been utilized to isolate the enzyme from hog spleen. The enzyme hydrolyzes "RNA core" readily at pH 6. However, no hydrolysis of tRNA with or without the 5′ phosphate has been observed. We are now varying conditions of temperature and pH in order to obtain digestion of tRNA.

References

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21.15 AGE-DEPENDENTS CHANGES IN ALCOHOL DEHYDROGENASE IN *DROSOPHILA*

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Introduction. — The alcohol dehydrogenase of *Drosophila* exists in five electrophoretic forms. In vitro experiments have shown that these five forms are the product of a single gene and can be converted from one to another by chemical means. This study is an examination of the in vivo conversion of enzyme forms as a function of age in the larva, pupa, and imago.

Results. — The quantity of alcohol dehydrogenase fluctuates in the larvae; at two days of age the amount of enzyme is twice that at either one or three days. After remaining relatively constant during further larval development and pupation, the enzyme again increases two- to threefold three days following emergence of the imago and remains at a high level for the subsequent two weeks.

The usual electrophoretic forms seen in *Drosophila* are ADH_5, ADH_3, and ADH_1. Of particular interest, ADH_2 appears for one day (day 3–4) in larval development, disappears in the pupa, and reappears about 24 hr after the imago emerges and persists until day 10–12. We interpret this to mean that physiological regulation of the alcohol dehydrogenase occurs, that a given form can be produced or removed, and that a regulatory process presumably exists in the *Drosophila* to accomplish this.

Reference

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21.16 ISOENZYMES: MECHANISM OF INTERCONVERSION

J. A. Knopp[1] K. Bruce Jacobson

It has been previously reported that one electrophoretic form of alcohol dehydrogenase from *Drosophila,* which is designated ADH_5, apparently forms a complex with NADH.[2] This complex was characterized by its quantum yield, which is 30 to 60 times greater than the separate components, and by the wavelength, 430 nm, corresponding to the maximum of fluorescence emission. We have continued to examine this formation of complex in order to compare with results from kinetic studies and to examine possible roles in the interconversion of the electrophoretic forms of this enzyme in the presence of NAD.[3]

By varying the concentrations of the two components, NADH and ADH_5, by 50-fold, the influence of the photochemistry of NADH became apparent. Fluorescence titrations under conditions where the instability of NADH to incident light can be minimized indicated a stoichiometry of 4 ± 1 as the ratio of NADH to ADH_5. This value agrees with the growing evidence that ADH_5 has four subunits. The dissociation constants determined from the same titration curves are in the range of 10^{-6} to 10^{-7} *M*. Enzyme kinetic measurements were made to determine the K_m for NADH, and the calculated K_m values were 1 to 2×10^{-7} *M*. The agreement between these two values of dissociation constants allows us to conclude that the fluorescence complex involves the interaction of NADH with the four binding sites of the enzyme.

This identification is relevant to the problem of interconversion because of the following observations. When NAD was added to ADH_5 under conditions similar to those employed to convert ADH_5 to ADH_1, a fluorescent signal was observed which increased in intensity with time. Part of this signal had the same spectral properties as the complex described above and disappeared upon heat denaturation of the enzyme. The rest of the signal was attributed to unbound NADH. An increase in absorbance at 340 nm was also observed in related experiments, and this increase corresponded to approximately 60 moles of NADH produced per mole of ADH_5 in the absence of any apparent substrates. We intend to explore the role of the NADH formation and the complex formation in the conversion of ADH_5 to ADH_1.

References

[1] Biophysics section.

[2] K. B. Jacobson, J. A. Knopp, and J. B. Murphy, *Biol. Div. Ann. Progr. Rept. Dec. 31, 1967,* ORNL-4240, p. 256.

[3] K. B. Jacobson and J. B. Murphy, *Biol. Div. Semiann. Progr. Rept. Jan. 31, 1967,* ORNL-4100, p. 159.

21.17 ISOENZYMES: THEIR REGULATION AND GENETIC CONTROL

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Alcohol dehydrogenase from *Drosophila melanogaster* exists in five or more forms, all of which are the product of a single gene. This study focuses on the ways in which these several forms of the enzyme are related and how they may be converted from one to another.

Earlier studies of this alcohol dehydrogenase showed that ADH_3 and ADH_1 were converted to ADH_5 during adsorption to DEAE-cellulose and that, conversely, ADH_5 was converted to ADH_3 and ADH_1 by exposure to NAD. We have examined the latter conversion process in some detail and have determined the optimal conditions of pH and concentration as well as finding inhibitors and stimulators. Furthermore, we found evidence for a change in protein conformation as ADH_5 is converted to ADH_1. This evidence is obtained from fluorescence studies. The fluorescence of the tryptophan residues in alcohol dehydrogenase decreases by tenfold when ADH_5 becomes ADH_1, and the fluorescence emission maximum shifts from 333 nm to 325 nm. This may be interpreted as a change in the tertiary structure in the vicinity of the tryptophan residues.

Physical and chemical studies have shown that the molecular weight is 60,500, the enzyme most likely consists of four subunits, and that each subunit has one tryptophan and one cysteine residue. The analysis for amino acids has been completed, and these values agree within 10% with the physically determined molecular weight.

Our view of the conversion processes is that NAD binds to the protein, possibly causing it to dissociate into subunits, and that a rearrangement of the tertiary structure occurs along with NAD binding. The single tryptophan in each subunit is involved in either the NAD binding or the rearrangement in protein conformation, or in both. We will continue to study the mechanism of conversion of ADH_5 to the other forms and compare these forms of the enzyme to these presumably same forms as they occur in the fly.

Reference

[1] Biophysics section.

21.18 STUDIES ON THE PHENYLALANYL-tRNA SYNTHETASES OF *NEUROSPORA CRASSA*

F. J. Kull K. Bruce Jacobson

Introduction. – The extreme specificity that aminoacyl-tRNA synthetases exhibit toward their respective tRNA's is of particular current interest because aminoacylation is considered the last protein-directed step of protein biosynthesis. The cytoplasmic phenylalanyl-tRNA synthetase of *Neurospora crassa* ($Syn^{Phe}_{N.\ crassa}$) can aminoacylate phenylalanine to heterologous (*Escherichia coli*) valine and alanine tRNA's ($tRNA^{Val\ (or\ Ala)}_{E.\ coli}$).[1] This unique heterologous aminoacylation affords an ideal system for the study of the mechanism of synthetase-tRNA interaction by comparison with homologous reactions. The advantages of this system are enhanced by use of highly purified tRNA's and synthetases. Studies on these heterologous reactions have led to three areas of research: (1) characterization of the multiple forms of $Syn^{Phe}_{N.\ crassa}$ found to occur in its cytoplasm, (2) isolation and stabilization of the major $Syn^{Phe}_{N.\ crassa}$, and (3) study of the mechanism of heterologous aminoacylation.

Results. – Three cytoplasmic $Syn^{Phe}_{N.\ crassa}$ activities (A, B, and C) have been demonstrated and separated by hydroxylapatite chromatography. The three activities retain their individual chromatographic properties throughout extensive rechromatography and long periods of storage. The three enzymes have different affinities toward purified $tRNA^{Val\ (or\ Ala)}_{E.\ coli}$ in Tris-Cl buffer. $Syn^{Phe}_{N.\ crassa}$ A and C have very similar sedimentation characteristics, which makes a monomer-dimer relationship unlikely.

$Syn^{Phe}_{N.\ crassa}$ C has been purified and isolated in a stable form, homogeneous after electrophoresis at several pH's. Purification was accomplished by ammonium sulfate fractionation, the use of hydroxylapatite, and DEAE-cellulose column chromatography.

Aminoacylation of phenylalanine to $tRNA^{Val}_{E.\ coli}$, catalyzed by $Syn^{Phe}_{N.\ crassa}$ C, is incomplete in Tris-Cl buffer but complete in cacodylate buffer.[2] Extremely low concentrations of inorganic pyrophosphate (10^{-8}–10^{-7} *M*) inhibit the rate of Phe-$tRNA^{Val}_{E.\ coli}$ formation in either buffer, but the extent of its formation is only affected by these pyrophosphate levels when carried out in Tris-Cl. Added inorganic pyrophosphatase increases the rate and extent of Phe-$tRNA^{Val\ (or\ Ala)}_{E.\ coli}$ formation in Tris-Cl, but only the rate in cacodylate. Inorganic pyrophosphate, which acts as a competitive inhibitor with respect to $tRNA^{Val}_{E.\ coli}$

in Tris-Cl, behaves as a noncompetitive inhibitor in cacodylate. Michaelis constants for $tRNA^{Val}_{E.\ coli}$ differ by a factor of 100, while K_i values differ only by 6 when compared in the two buffer systems. These differences indicate an important role for inorganic pyrophosphate in heterologous aminoacylation reactions carried out in Tris-Cl. Experiments are in progress to further examine the mechanism of aminoacylation.

References

[1] W. E. Barnett and K. B. Jacobson, *Proc. Natl. Acad. Sci. U.S.* **51**, 642 (1964).

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21.19 INTERACTIONS OF *E. COLI* VALINE tRNA WITH *N. CRASSA* PHENYLALANYL-tRNA SYNTHETASE

P. O. Ritter K. Bruce Jacobson

Phenylalanyl-tRNA synthetase from *N. crassa* ($Syn^{Phe}_{N.\ crassa}$) reacts with valine tRNA from *E. coli* ($tRNA^{Val}_{E.\ coli}$) to give Phe-$tRNA^{Val}_{E.\ coli}$. This reaction is of considerable interest since the phenylalanine enzyme recognizes valine tRNA, a clear case of misrecognition. By studying this reaction it may be possible to gain some insight into the mechanism of enzymatic aminoacylation and to learn something about enzyme recognition sites on tRNA.

In Tris-HCl buffer under optimal assay conditions, the final yield (yield at the time net aminoacylation has ceased) of Phe-$tRNA^{Val}_{E.\ coli}$ is a function of enzyme concentration, and submaximal charging is obtained even when equimolar amounts of enzyme and tRNA are used. No net aminoacylation occurs after 60 to 90 min regardless of the enzyme concentration and the final yield. At low enzyme concentrations, 20% dimethyl sulfoxide leads to a greater than tenfold increase in final yield. Ethanol (10%) also increases the final yield, while NaCl, NH_4Cl, and 2-mercaptoethanol lead to a decreased final yield.

If K-cacodylate buffer (pH 6.3) is used in place of Tris-HCl buffer (pH 8.1), there is a marked increase in the final yield obtained at a given enzyme concentration and a change in the overall kinetics of the aminoacylation reaction. In no case does net aminoacylation stop within 120 min unless all of the $tRNA^{Val}_{E.\ coli}$ has been charged. Dimethyl sulfoxide (20%) inhibits charging in K-cacodylate buffer, but lower levels are slightly stimulatory. Ethanol, 2-mercaptoethanol, NaCl, and NH_4Cl are inhibitory at all concentrations.

The large difference in the pH optimum in Tris-HCl (pH 8.1) and K-cacodylate (pH 6.3) buffers emphasizes the fact that one or both of the buffers are doing more than just maintaining a relatively constant pH in the reaction mixture. Although the pH optimum is considerably higher in the Tris-HCl buffer, nonenzymatic hydrolysis is probably not the main factor that determines the final yield of Phe-$tRNA^{Val}_{E.\ coli}$ in this buffer, since the half-life of Phe-$tRNA^{Val}_{E.\ coli}$ under conditions of Phe-$tRNA^{Val}_{E.\ coli}$ formation is approximately 1 hr.

Low levels of K-cacodylate (pH 8.0) slightly stimulate aminoacylation in Tris-HCl (pH 8.1), but concentrations above 20 m*M* are inhibitory. All levels of Tris-HCl (pH 6.3) inhibit aminoacylation in K-cacodylate buffer (pH 6.3).

$Syn^{Phe}_{N.\ crassa}$ is unable to place valine on $tRNA^{Val}_{E.\ coli}$, but the enzyme can deacylate Val-$tRNA^{Val}_{E.\ coli}$ in Tris-HCl buffer under conditions of Val-$tRNA^{Val}_{E.\ coli}$ formation. Thus $Syn^{Phe}_{N.\ crassa}$ can apparently recognize Val-$tRNA^{Val}_{E.\ coli}$.

The results discussed above emphasize the important role that the general environment of an enzyme plays in determining the activity of this enzyme. There are many published accounts of "abnormalities" associated with heterologous aminoacylation reactions, and it is possible that some of these "abnormalities" were a result of the buffer which was used. The large effect of environment on enzymatic activity also raises the question as to whether the activity exhibited by an enzyme under in vitro conditions at all resembles in vivo activity. Would an error in recognition be made if $Syn^{Phe}_{N.\ crassa}$ and $tRNA^{Val}_{E.\ coli}$ existed together in vivo?

21.20 A COMPARATIVE STUDY OF THE tRNA's OF A WILD AND A SUPPRESSOR STRAIN OF VERMILION EYE COLOR IN *DROSOPHILA MELANOGASTER*

Daniel R. Twardzik K. Bruce Jacobson

Introduction. – Beadle and Ephrussi in 1936 found a specific suppressor of vermilion eye color in *Drosophila*, the locus of which maps on the (X) sex chromosome. These authors also demonstrated that the suppressor acted by partially restoring the ability of the mutant to produce kynurenine.

Many studies of the mechanism of suppression have indicated that tRNA, activating enzymes, and other components of the translation process may be involved.

Suppressors of amber and ocher mutations in microorganisms permit the propagation of the polypeptide chain beyond the site of the primary genetic lesion by causing the insertion of an amino acid at the site of the amber (or ocher) triplet. A base change in the anticodon region of a tRNA molecule may allow it to function in the mechanism of suppression.

Results. – A comparison of various tRNA's of a wild strain and a mutant strain of *Drosophila* carrying the suppressor mutation, designated su(v), was made. Transfer RNA was isolated by the phenol method; synthetases were prepared utilizing a 105,000 × *g* supernatant, as ribonuclease activity was extensive by other methods. Enzyme preparations were unstable in 30% propylene glycol or 10% glycerol at liquid-nitrogen temperatures, whereas storage in 50% glycerol at the same temperatures gave long-term stability. Double labeling techniques were combined with a reverse-phase-chromatography "2" system to compare the profiles of phenylalanine, leucine, serine, and tyrosine tRNA's. Optimum charging conditions (pH, ATP, and Mg concentration) were determined for each amino acid studied.

Four isoaccepting forms of leucine tRNA were found, while all other tRNA's studied chromatograph as a single peak. Cochromatography of tyrosine tRNA gave a 30-ml displacement of peak fractions, with the Su(v) tyrosine tRNA eluting at a lower salt concentration than normal tyrosine tRNA. To determine if any component of the enzyme preparation was modifying the tyrosine tRNA, wild-type enzyme was used to charge Su(v) tyrosine tRNA, and su(v) enzyme was used to charge wild tRNA. Charged tyrosine tRNA's from the enzyme crosses were then cochromatographed with charged tyrosine tRNA's from homologous systems. In both cases, the wild and the Su(v) tyrosine tRNA still exhibited different peak positions. Melting of both tRNA's at 100°C for 6 min followed by quick cooling before charging did not affect the difference in column profiles. This indicated that configurational differences between the two tyrosine tRNA's were not the cause for their observed displacement on RPC "2" columns.

DEAE cochromatography of T_1 RNase digests of labeled tyrosine tRNA from both wild and mutant strains are in progress to compare the 3′-terminal oligonucleotides.

Genetic studies are also in progress to determine if the observed difference in tyrosine-tRNA is due to the suppressor gene or to gene located on an autosomal chromosome.

22. Nucleic Acid Chemistry

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22.1 STRUCTURE AND FUNCTION OF $tRNA^{Phe}$

Mayo Uziel
H. G. Gassen
A. Jeannine Bandy
Chongkun Koh

The sequence of phenylalanine tRNA isolated from *E. coli* B has been determined (Fig. 22.1.1). The earlier conclusions about portions of the sequence have been confirmed and extended by the isolation of large fragments of the molecule after partial enzymatic hydrolysis and chemical modification. Chromatography of the RNase T_1 hydrolysate of the RNA remaining after sequential degradation through 26 cycles showed the disappearance of several oligonucleotides.[1] Combination of the base analysis data and the expected oligonucleotides from this end of the molecule permitted construction of the partial sequence -U-m^7G-C-C-U-U-G-G-T-ψ-C-G-A-U-U-C-C-G-A-G-U-C-C-G-G-G-G-C-A-C-C-A. This was confirmed by isolation of the nonadecanucleotide A-U-U-C-C-G-A-G-U-C-C-G-G-G-C-A-C-C-A from partial T_1 hydrolysis. The intact anticodon region, including the supporting base-paired region, was also isolated from partial RNase T_1 hydrolysates. By analogy to other tRNA molecules and because of the oligonucleotide overlap requirements, the latter two segments could be put together to form a sequence 51 residues long: A-G-G-G-G-A-ψ-C-C-G-U-m^7G-C-C-U-U-G-G-T-ψ-C-G-A-U-U-C-C-G-A-G-U-C-C-G-G-G-C-A-C-C-A.

We were not successful in the isolation of large fragments from the remaining region of the molecule by controlled enzymatic hydrolysis, so we turned to chemical modification. Selective reduction with $[^3H]NaBH_4$ followed by enzymatic hydrolysis gave a number of labeled oligonucleotides. The isolation of the U-labeled oligonucleotide G-A-hU* from pancreatic RNase hydrolysates confirmed our identification of the

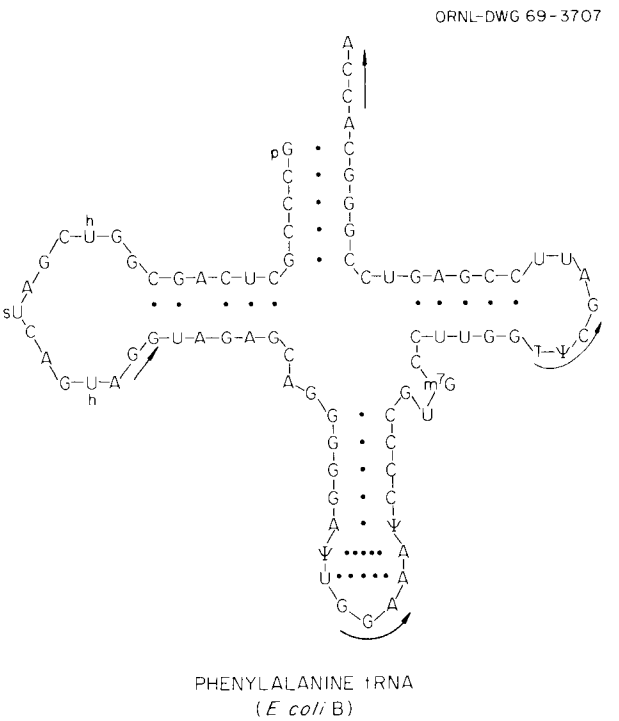
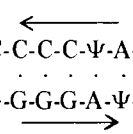


Fig. 22.1.1. Sequence of *E. coli* B Phenylalanine Transfer RNA.

sulfur-containing region as -G-A-sU-C(C)AG-. The combined data of the oligonucleotides isolated from exhaustive enzymatic hydrolysis and the sequences of the large fragments permitted reconstruction of the sequence illustrated in Fig. 22.1.1 (in the cloverleaf representation). The questions raised in the previous report[2] about the relationships of tRNA structure to function are still pertinent.

In addition, one other question may be raised. Construction of models of the anticodon region shows that several trinucleotide sequences may form the intermolecular base-paired structure. In all the other known tRNA sequences, the most favored position contains the expected anticodon. In phenylalanine tRNA, this position is occupied by the wholly unexpected sequence GGA. Although it is theoretically possible for this sequence to respond to the known anticodons, it is necessary to assume a new degeneracy mode. The combination of this anomaly with the unusual potential interchain base pairing



may account for this anomalous anticodon region.

The expected anticodon, on the other hand, is found overlapping the above trinucleotide sequence, so that a small conformational adjustment in the synthetic complex could permit the normal base pairing observed in the translation process.

References

[1] This report, paper 22.2.

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22.2 SEQUENTIAL DEGRADATION OF NUCLEIC ACIDS

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The value of a sequential degradation procedure depends upon the number of times the process may be used before the interpretation of the data becomes impossible. We have applied the procedure described by Khym and Uziel[2] to the purified phenylalanine tRNA and have carried it through 26 cycles. The first 19 cycles yield interpretable data in that only one base appears to be located at each position. The remaining data are obscured by the continued mechanical loss and by the poorly understood reaction of periodate with pseudouridine.

The correctness of the interpretation of the first 19 cycles has been confirmed by isolation of this fragment from partial enzymatic hydrolysates.[3] This is the first time that a substantial portion (~25%) of a nucleic acid has been directly sequenced by a method independent of selective enzymatic hydrolysis and has been confirmed by the latter approach to sequence determination. Analysis of the quantitative data shows that the yields of RNA recovered are greater than 98% per cycle where at least ten manipulations are performed per cycle. Thus the theoretical limitation on the number of cycles that may be performed is dependent primarily upon the amount of starting material.

The practical limitations lie in the solubility of the tRNA·CTA complex and in the mechanical losses and asynchrony introduced by the centrifugal separation and incomplete enzyme removal respectively.

Analysis of the des(ω – 26)tRNA^{Phe} (ref. 4) by the procedures of Uziel and Gassen yielded the expected oligonucleotides from the residual chain, except for the oligonucleotide containing the minor base 7-methylguanine. This absence is consistent with the 26 cycles of degradation and the sequence proposed for *E. coli* B tRNA^{Phe} by Uziel and Gassen.[3]

References

[1] Nucleic Acid Enzymology section.

[2] J. X. Khym and M. Uziel, *Biochemistry* 7, 422 (1968).

[3] M. Uziel *et al.*, this report, paper 22.1.

[4] The term describes the RNA chain (originally ω in length) remaining after 26 cycles of sequential degradation.

22.3 NUCLEIC ACID SEQUENATOR

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The major losses in the sequential degradation of phenylalanine tRNA were due to mechanical entrapment during decantation after the centrifugation steps.[2] The utilization of a filtration process should reduce these losses to a minimum and as a bonus permit a more rapid separation of the insoluble RNA·CTA complex. We have designed and constructed a reaction vessel that will accommodate a charge of up to 30 mg of RNA. This contains ports for reagent addition, a magnetic stirring device which does not cause foaming, and a combined filter and stirrer support which minimizes the dilution on change of solvents and reagents through the

lower port. There is complete recovery of the precipitate in control experiments using periodate-treated *E. coli* B tRNA. The ultraviolet absorbance of the supernatant fluid is the same as the filtered reagent blank.

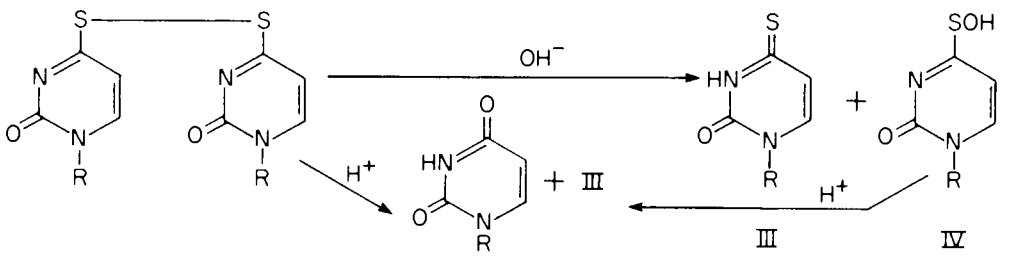
References

[1] Instrumentation and Controls Division.
[2] M. Uziel *et al.*, this report, paper 22.2.

22.4 THE PROPERTIES OF PYRIMIDINE DISULFIDES

B. C. Pal

The recent discovery of 4-thiouridylic acid in the soluble *E. coli* RNA[1] and the formation of the disulfide bond therein by oxidation[2] have stimulated interest in the chemistry of bis(4-thiouridine) disulfide (I). Conflicting reports[2,3] on the products of alkaline hydrolysis of I prompted a further investigation, using the 1-methyl analog (II), synthesized by I_2 oxidation of 1-methyl-4-thiouracil (III). It has been established that both I and II hydrolyze almost quantitatively in alkali to the corresponding thione (III) and sulfenic acid (IV) in 1:1 molar ratio. 1-Methyluracil-4-sulfenic acid has been isolated and characterized as its silver salt. The uridine-4-sulfenic acid has been identified by the similarity of its uv absorption spectra with those of its 1-methyl analog. 1-Methyluracil-4-sulfenic acid is more unstable in acid than in alkali. In hot 1 *N* HCl, it undergoes quantitative transformation to 1-methyluracil and 1-methyl-4-thiouracil in 2:1 molar ratio. On the other hand, the disulfides I and II hydrolyze in acid to the corresponding thiones and uracil derivatives quantitatively in 1:1 molar ratio without going through the sulfenic acid intermediate.



I. R = β-D-RIBOSYL
II. R = METHYL

We have been unable to find any evidence for the formation of a single product [T] upon treatment of I with alkali, as reported earlier by Lipsett.[2] 4-Thiouridine disulfide shows no appreciable fluorescence in alkali, also in conflict with the earlier report.[2] Uridine-4-sulfenic acid shows the positive Cotton effect expected of all β-nucleosides.[4] The properties of the sulfenic acid formed on alkaline hydrolysis of oxidized 4-thiouridine (I) may help to explain difficulties in recovering sulfur-containing nucleotides from tRNA's.

References

[1] M. N. Lipsett, *J. Biol. Chem.* **240**, 3975 (1965).
[2] M. N. Lipsett, *J. Biol. Chem.* **242**, 4067 (1967).
[3] M. Uziel, *Biochem. Biophys. Res. Commun.* **25**, 105 (1966).
[4] T. R. Emerson *et al.*, *Biochemistry* **6**, 843 (1967).

23. Nucleic Acid Enzymology

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23.1 SOME PROPERTIES OF BACTERIOPHAGE T2 mRNA

Elliot Volkin W. S. Riggsby M. Helen Jones

We are applying procedures of phenol extraction, MAK column chromatography, and a column DNA hybridization method to isolate purified, labeled mRNA species. The mRNA's found after phage T2 infection are being investigated by sedimentation studies and end-group analyses to determine (1) average size and (2) average nucleotide compositions of both the 3′ and 5′ termini. Such data are important in evaluating the nature of the many proteins induced in this system.

Over 90% of the mRNA is insoluble in 2 *M* NaCl and thus easily separable from tRNA and DNA. MAK column chromatography and sucrose gradient centrifugation demonstrate that a number of T2 mRNA species can be separated from the ribosomal RNA. The average size of the total mRNA formed either "early" or "late" after infection is about 600 nucleotides. As expected, the mRNA sediments in a heterogeneous fashion with a "peak" at about 11 to 12S. The mRNA can be separated into a minimum of nine separable fractions by MAK column chromatography. These fractions vary in average size from about 120 to about 900 in nucleotide lengths. There is a reasonably good correspondence between these values and sedimentation rates. Specific hybridization to, and elution from, T2 DNA results in the virtual removal of ribosomal RNA and a very high degree of purification of the mRNA. However, studies on this product indicate that it may have become somewhat degraded or its secondary structure altered, or both, during the hybridization-elution.

23.2 MODIFICATION OF *EUGLENA* tRNA's BY LIGHT AND CYTOPLASMIC MUTATION

W. E. Barnett C. J. Pennington, Jr.
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The tRNA's of *Euglena* have been examined by reversed-phase column chromatography (RPC-II), and the following observations have been made: (1) Exposure of dark-grown cells to light results in the synthesis of a new species of tRNA for each of several amino acids. (2) Subsequent growth in the dark results in the loss of the light-induced species. (3) A cytoplasmically

inherited bleached mutant has tRNA's similar to dark-grown wild-type cells, whether grown in the light or the dark.

These results raise the interesting possibility that cellular synthesis of the functional photosynthetic apparatus is triggered by the induction of a unique set of tRNA's by light; this is the subject of our current research.

Reference

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23.3 FRACTIONATION OF CYTOPLASMIC AND MITOCHONDRIAL tRNA's OF *NEUROSPORA CRASSA*

J. L. Epler R. A. Dolbeer

Amino-acid-specific transfer RNA's can be highly purified by countercurrent distribution. We have surveyed the behavior of the tRNA's from whole-cell extracts of the fungus *Neurospora crassa* in the system of Holley. Many isoaccepting forms are separable, but their origin and function in the fungal cell are complicated by the observation that the mitochondria of this organism contain tRNA's and aminoacyl-tRNA synthetases in addition to those found in the cytoplasm. These mitochondrial tRNA's were found to be distinct from those of the cytoplasm in acylation specificities and coding properties. A preliminary report from Novelli's group has shown that the mitochondrial seryl-, leucyl-, and methionyl-tRNA's are chromatographically distinct. We have extended this work and used reversed-phase chromatography (RPC-2) to show distinct and separable species of mitochondrial and cytoplasmic tRNA's for all the amino acids. Countercurrent distribution of isolated mitochondrial tRNA was also used to clarify the relationship of the organelle tRNA's to their cytoplasmic counterparts. The differences in these translational macromolecules supply additional evidence for a unique protein-synthesizing system in *Neurospora* mitochondria.

23.4 tRNA's FROM DIFFERENT DEVELOPMENTAL STAGES OF RHYNCHOSCIARA

S. A. Fairfield[1] Ellen Mattingly[2]
W. E. Barnett C. L. Parker[2]

The sciarid fly *Rhynchosciara* has a well-defined life cycle with discrete developmental stages in which large quantities of eggs, larvae, and pupae are well synchronized.

Transfer-RNA's from these different stages have been compared using reversed-phase column chromatography (RPC-II), and quantitative as well as qualitative differences for several amino acid tRNA's have been found.

Investigation into the possibility that these new species are related to developmental stage is continuing.

References

[1] Student at the UT–Oak Ridge Graduate School of Biomedical Sciences.
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23.5 BIOCHEMICAL ANALYSIS OF ^{32}P-LABELED REPAIRED SECTIONS OF BACTERIOPHAGE T5 DNA

R. K. Fujimura

Biochemical studies on the end-to-end joining of preexisting DNA strands are being undertaken to get a more detailed understanding of the mechanism of formation of repaired DNA and recombinant DNA. Currently the experiments are being performed to test the hypothesis that the repair process of preexisting strands involves the de novo synthesis of a short stretch of oligonucleotides to fill the gap between DNA fragments. In the first set of experiments,[1] bromodeoxyuridine was incorporated into preexisting bacteriophage DNA inside the host bacteria in the absence of DNA replication. The manner of incorporation was consistent with the mechanism that single-strand breaks yield fragments phosphorylated at the 5′ end of the broken point. In the rejoining process, 5′ nucleotides are incorporated to fill the gap between DNA fragments. In the next set of experiments, which are still in progress, bacteria were grown in a ^{32}P-containing medium and then at 43° infected with a temperature-sensitive mutant T5ts53 labeled with dBrUrd. DNA was extracted and purified by alkaline CsCl gradient equilibrium centrifugation, followed by annealing to T5 DNA-nitrocellulose powder. This DNA preparation is as pure as DNA from purified T5 phage, as judged by annealing to T5 DNA-nitrocellulose disks. When the DNA was degraded to 5′ and 3′ nucleotides, only the 3′ and not the 5′ dBrUMP was ^{32}P labeled. This is consistent with the data obtained in the first set. The ^{32}P-labeled section of this DNA has a base composition similar to the whole T5 DNA. The average chain length of the ^{32}P-labeled section is tentatively estimated to be about 30 and is independent of the time of harvest.

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23.6 CHANGE IN DISTRIBUTION COEFFICIENTS OF PURINES AND PYRIMIDINES BY PRETREATMENT OF SEPHADEX COLUMNS

J. X. Khym

In anticipation of using Sephadex G-10 to desalt purine and pyrimidine samples, the distribution coefficients (K_D) of these compounds were determined under a variety of conditions. The pH of the sample placed on the Sephadex columns influences somewhat the K_D's of all the compounds studied. However, a very remarkable change in the K_D's of cytosine, adenine, and guanine is brought about simply by pretreatment of the Sephadex columns. For instance, the K_D of cytosine in water or dilute salt is about 2. This can be changed to a K_D of greater than 15 when a Sephadex column is prerinsed with dilute HCl and then with water to neutral pH before the cytosine sample is added to the column.

The mechanism involved in changing these K_D's is probably that of ion exchange. Sephadex has some carboxyl groups throughout its three-dimensional dextran matrix. At low pH, these carboxyl groups would be in their acid form and would stay that way even if a column were water washed. Such a column would then act like a cation exchanger and hence tend to retain adenine, cytosine, and guanine, whose amino groups could attract a proton from the carboxyl groups and become positively charged and then become ionically bound to the now negatively charged carboxyl groups. This property of Sephadex is being explored to see whether it can be used to advantage in separating and desalting purine and pyrimidine samples.

23.7 SEQUENTIAL NUCLEOTIDE ANALYSES

J. X. Khym Mayo Uziel[1]

The "cetyltrimethylammonium (CTA) precipitation method"[2] involves various solubilizations and precipitations in each cycle of the procedure. These manipulations give rise to two major problems after several cycles have been carried out. One difficulty is the small loss of RNA that is associated with each precipitation step and which on an accumulative basis becomes an important factor as the number of cycles progresses. The other difficulty involves the complete removal of enzyme (after phosphatase action), which is necessary so that it will not carry over to the oxidation-elimination step and allow a release of small amounts of the penultimate base of each cycle and hence cause asynchrony in the stepwise degradation procedure. Two changes in the CTA degradation procedure were made to minimize these problems.

Solubility losses were lowered by using CTA at a concentration of 20 micromoles/ml in place of water and lysine monohydrochloride in place of sodium chloride, at each precipitation and solubilization and washing step of the procedure. Phosphatase enzyme activity should be lowered in the presence of ethylenediaminetetraacetic acid (EDTA). Therefore, to combat the problem of asynchrony, EDTA was added at the oxidation-elimination step. Evaluations of these changes in the CTA sequence procedure are being tested.

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23.8 ASCORBIC ACID SULFATE

C. G. Mead

Undeveloped cysts of the brine shrimp *Artemia salina* contain a sulfur derivative of ascorbic acid. The properties of this compound are consistent with those expected of a derivative of ascorbic acid sulfated in either the 2 or 3 position. Speculation as to the possible existence of this compound and its biological function as a sulfating agent has appeared in the literature because of a number of indirect relationships between ascorbic acid deficiency and sulfate incorporation into biologically important compounds.

The compound can be isolated in pure form from an acid-soluble extract by column chromatography on Dowex 1 followed by DEAE. Aqueous solutions of the compound are stable for several days at room temperature over a range of pH's from 1 to 12. The ultraviolet absorption maxima of the compound are at 230 mμ at pH 1 and at 254 mμ at pH 8. Acid hydrolysis results in a product whose properties are identical to those of ascorbic acid as judged by ultraviolet absorption spectra, the appearance of reducing power, optical rotation, paper chromatography, and column chromatography. Elemental analysis of the compound indicates the formula to be $C_6H_{14}O_9SN_2 \cdot H_2O$. The compound is an acid sulfate and exists in the lactone form as judged by infrared spectrophotometry. The position of the sulfate must be either on carbon 2 or 3, because only substitutions of these vicinal enolic groups result in nonreducing derivatives of ascorbic acid.

23.9 STABILITY OF OLIGORIBONUCLEOTIDE-DENATURED DNA COMPLEXES[1]

S. K. Niyogi

It has previously been shown[2] that in the absence of Mg^{2+} ion a chain length of ten or more ribonucleotides is required to form a ribonuclease-resistant complex with denatured DNA. Even these short oligonucleotides display a high degree of specificity for species; oligonucleotides produced from T-even phage DNA's will form complexes only with T-even DNA's, not with T-odd DNA's and vice versa. In the presence of 0.01 *M* $MgCl_2$, T7 RNA oligonucleotides as small as 8-mers can form a detectable complex with denatured T7 DNA; 9-mers and 10-mers form complexes with increasing efficiency. Small yet consistent values were obtained when 8- to 10-mers from T7 RNA were tested for their annealing to denatured T2 DNA, with the values falling off rapidly with increasing chain length. The expression of heterology between the T-even and T-odd phage DNA's seems to occur on passing from a chain length of 9 to 10.

The stability of homologous oligoribonucleotide-denatured DNA complexes on nitrocellulose filters was determined by measuring the temperature of melting of the oligoribonucleotide from the DNA filters. The melting temperatures (in 2X SSC) of 13- to 16-mers from T2 RNA are 40, 45, 51, and 57° while those of 12- to 17-mers from T7 RNA are 62, 65, 69, 72, 76, and 79°. T3 and T4 oligonucleotides gave results very similar to T7 and T2 oligonucleotides respectively. Thus the melting temperature increases with increasing chain length of the oligonucleotide, but the T-odd oligonucleotides melt at temperatures considerably higher than T-even oligonucleotides of the same chain length. Evidently, T-odd oligonucleotides having higher G + C contents form complexes that are more stable than those formed by T-even oligonucleotides.

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23.10 EFFECT OF TERMINAL NONCOMPLEMENTARY BASES ON THE STABILITY OF OLIGONUCLEOTIDE-POLYNUCLEOTIDE COMPLEXES

S. K. Niyogi

Poly AU, poly AC, and poly AG (all ^3H labeled and having 1:1 base ratios) were prepared by using polynucleotide phosphorylase. Oligonucleotides of types $(Ap)_nUp$ and $(Ap)_nCp$ were obtained by the action of pancreatic ribonuclease on poly AU and poly AC respectively. Oligonucleotides of type $(Ap)_nGp$ were obtained by the action of ribonuclease T1 on poly AG. Each oligonucleotide was annealed to poly U, and the stability of such a complex was measured by thermal chromatography on hydroxylapatite columns.[1]

For chain lengths longer than 4, oligonucleotides $(Ap)_nUP$, $(Ap)_nCp$, and $(Ap)_nGp$ have melting temperatures close to those for $(Ap)_n$; this suggests that the noncomplementary base does not interact with poly U. Removal of the 3′-phosphate does not affect the melting temperature. At a chain length of 4, there is some positive interaction since $(Ap)_3Up$, $(Ap)_3Cp$, and $(Ap)_3Gp$ all form stronger complexes with poly U than does $(Ap)_3$, as indicated by higher melting temperatures. On removal of the 3′-phosphate the melting temperatures of $(Ap)_3C$ and $(Ap)_3G$ drop by a few degrees, while that of $(Ap)_3U$ remains the same. At the trinucleotide level, $(Ap)_2Up$ melts at the same temperature as $(Ap)_2$, while $(Ap)_2Cp$ and $(Ap)_2Gp$ have slightly higher melting temperatures. Upon removal of the 3′-phosphate, the melting temperatures of $(Ap)_2C$ and $(Ap)_2G$ remain the same, while that of $(Ap)_2U$ sharply drops from 12 to 5°C. The reasons for this sharp change are not clear. At the level of dinucleotides the noncomplementary base does not interact with poly U, since these oligonucleotides did not form complexes with poly U.

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23.11 EFFECT OF TEMPERATURE ON THE OLIGONUCLEOTIDE STIMULATION OF THE RNA POLYMERASE REACTION[1]

S. K. Niyogi Pamela E. Wilton

It has been shown[2] previously that complementary oligonucleotides with a free 3′-hydroxyl end stimulate (by acting as primers or chain initiators) the RNA polymerase reaction with synthetic polyribonucleotides as templates. In the present studies, both adenine and uracil oligonucleotides of various chain lengths were tested for their stimulatory activity at various temperatures as a means of studying the interaction of the oligonucleotides with the template polymer. For uracil oligonucleotides of chain length 2 to 6, the temperatures for maximal stimulation of poly U formation

(with poly A as template) are 34, 37, 40, 43, and 46°C respectively. For adenine oligonucleotides of chain length 2 to 6, the temperatures for maximal stimulation of poly A formation (with poly U as template) are 34, 37, 40, 43, and 50°C respectively. The optimum temperatures for poly U and poly A formation in the absence of stimulatory oligonucleotide are 31 and 34°C respectively. The above temperature studies suggest that there is an interaction between the oligonucleotide and the template to form a complex that is sensitive to temperature; oligonucleotides with longer chain lengths form complexes that are more stable at higher temperatures. For the same chain length, the optimum temperature of stimulation for the adenine oligonucleotide is similar to that for the uracil oligonucleotide. This further suggests that the mode of stimulation involves the interaction of the oligonucleotides with the complementary polynucleotides.

It is interesting to note that, with the exception of the di- and trinucleotides, the melting temperature of each oligo A• poly U complex (as measured by thermal chromatography on hydroxylapatite at an ionic strength similar to that of the enzymic reaction) is very similar to the oligonucleotide's optimum temperature of stimulation. The temperatures for maximal stimulation by the di- and trinucleotides are considerably higher than their melting temperatures. Apparently the RNA polymerase stabilizes the complexes formed by these shorter oligonucleotides with the complementary polynucleotide.

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24. Developmental Biochemistry

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24.1 PURINE NUCLEOTIDE INTERCONVERSION AND REGULATION OF DNA SYNTHESIS IN *ARTEMIA SALINA*

F. J. Finamore Rose P. Feldman

Recently we reported[1,2] that larvae of the brine shrimp *Artemia salina* are incapable of synthesizing purines *de novo* and that they convert guanine derivatives to adenine compounds as they are required by the organism. Furthermore, we presented evidence that P^1,P^4-diguanosine 5′-tetraphosphate functions as a principal source of DNA adenine when nauplii are undergoing rapid DNA synthesis.[2]

To determine if the purine and phosphate components of diguanosine tetraphosphate enter DNA as a unit, we exposed nauplii to both ^{3}H-guanosine and ^{32}Pi and measured the $^3H{:}\alpha^{32}P$ ratio of ATP, diguanosine tetraphosphate, and 5′-AMP derived from both RNA and DNA. We observed that the $^3H{:}\alpha^{32}P$ ratios of ATP approximate those found in 5′-AMP of both RNA and DNA, whereas the corresponding ratios of diguanosine tetraphosphate were considerably higher than those found in 5′-AMP of either nucleic acid. These results indicate that if indeed diguanosine tetraphosphate functions as the major source of DNA adenine, as our previous results indicate,[2] then its purine and phosphate components cannot be incorporated *directly* into DNA as a unit but must first pass through the main nucleotide pool of the organism.

In an attempt to gain some insight into the mechanism(s) by which guanine derivatives are converted to adenine compounds, the following pulse-chase experiment was performed. Nauplii were exposed to ^{3}H-guanosine for 30 min then transferred to a medium containing a large excess of unlabeled guanosine. At 1-hr intervals thereafter, we determined the specific activities of the acid-soluble nucleotides and purines from RNA and DNA. We observed that after 30 min exposure to the isotope, the compounds of highest specific activity were GTP and GMP, although each of the other acid-soluble nucleotides was labeled to a significant extent. Of considerable interest, however, was the remarkable decrease that occurred in the specific activities of GTP and GMP when the nauplii were placed in unlabeled medium for only 1 hr. In contrast, no such decrease was evident in the specific activities of ADP, ATP, or diguanosine tetraphosphate. Further, the large decrease in specific activities of GTP and GMP was not accompanied by a corresponding increase in specific activities of other nucleotides or of the purines obtained from either RNA or DNA. Upon investigating the external medium, we found that the decrease in specific activities of GTP and GMP could be accounted for by loss of ^{3}H-guanosine from these compounds to the external environment. From these results it appears that ^{3}H-guanosine is rapidly incorporated and converted to GMP and GTP by nauplii of *Artemia* but that only a small quantity of each enters the metabolic pool of the organism; the major portion of ^{3}H-GMP and ^{3}H-GTP is reconverted to ^{3}H-guanosine and is lost to the medium. Thus at least two distinct pools of guanosine, GMP, and GTP may exist: one that represents the bulk of these compounds and participates in nucleic acid synthesis and nucleotide interconversion and another smaller pool that exchanges with the environment.

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24.2 CHARACTERIZATION OF AN ENZYME RESPONSIBLE FOR CDP:CHOLINE FORMATION IN *ARTEMIA SALINA*

R. D. Ewing

Extracts of nauplii of *Artemia salina* contain a particulate enzyme which converts CMP to a compound identified as cytidine diphosphocholine. This reaction is completely dependent upon magnesium ions and is not affected by additions of ATP, UTP, CTP, GTP, diguanosine triphosphate, or diguanosine tetraphosphate. CTP will not replace CMP in this reaction, and addition of phosphocholine to the reaction has no effect, indicating that the enzyme is not CTP-phosphocholine cytidylyl transferase. The source of choline in this reaction has not been determined, but upon addition of suitable detergents, the reaction can be stimulated by addition of lecithin. These observations suggest that the enzyme may be a form of CDP-choline-1,2-diglyceride choline phosphotransferase in which the equilibrium is shifted strongly in favor of CDP-choline formation. The source of energy necessary for the formation of the pyrophosphate bond remains unclear. Attempts to solubilize the enzyme and separate it from associated lipids in order to further characterize the reaction are being carried out at the present time.

25. Biochemical Regulation

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25.1 REGULATION OF ENZYME TURNOVER

F. T. Kenney L. E. Roberson

Little is known of the mechanisms responsible for the vastly different rates at which specific proteins undergo turnover in mammalian tissues. Turnover of the soluble tyrosine transaminase is about 25 times faster than the bulk of the soluble proteins of rat liver. In common with the turnover of other enzymes, that of tyrosine transaminase requires energy.[1] However, the turnover of this enzyme is blocked when protein synthesis is directly inhibited by cycloheximide[2] or indirectly by actinomycin;[3] this response is not common to other liver enzymes, including those which undergo turnover at a comparable rate. To account for this requirement for protein synthesis in turnover of tyrosine transaminase, we have proposed three possibilities: (1) the ribosomal mechanism for synthesis of this enzyme can be run in reverse, (2) a specific protein which must be continuously synthesized is involved in transaminase turnover, (3) cessation of protein synthesis permits the accumulation of a substance which stabilizes the enzyme against denaturation and thereby against degradation.

The first of these seems unlikely (but not impossible) from a consideration of the complexity of the molecular interactions involved in the synthesis of proteins. The second is discomforting in that it suggests an infinite continuum of proteins, each being required to degrade the next; in addition, the hypothetical "degrading enzyme" would itself have to undergo turnover extremely fast (half-life of a few minutes) to account for our data. The third alternative would thus seem to be the best choice.

To investigate the possibility of stabilizers of the transaminase accumulating when protein synthesis is blocked, we prepared soluble liver extracts from untreated control rats and from rats treated for several hours with enough cycloheximide to block protein synthesis completely. Rates of denaturation of tyrosine transaminase in these extracts during mild heating were compared under a variety of conditions. Under none of the experimental conditions chosen were we able to detect significant differences in denaturation rates which could be attributed to cycloheximide treatment.

This result could mean that the "stabilizer" concept is incorrect or that in vitro denaturation studies do not provide an adequate model for the turnover process

occurring in vivo. These studies will be continued and will be expanded to include investigation of the possibility that hormones play a role in regulating turnover.[4]

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25.2 REGULATION OF HEPATIC ENZYME SYNTHESIS BY CYCLIC AMP

W. D. Wicks Kai-Lin Lee F. T. Kenney

It has been known for several years that glucagon elevates the activity of a few enzymes in rat liver. When it was discovered that cyclic AMP mediated the induction of tyrosine transaminase by glucagon,[1] it was of obvious importance to see if other glucagon-inducible enzymes were also influenced by the cyclic nucleotide. The organ culture system of fetal rat liver provided an ideal experimental situation in which direct action of inducers could be monitored.

Because of its well-known response to glucagon in vivo,[2] phosphoenolpyruvate carboxykinase (PEPCK) was assayed in the culture system in the presence and absence of cyclic AMP. Within 1 hr after addition of cyclic AMP, there was a detectable increase in PEPCK activity. Enzyme activity continued to rise for several hours, ultimately reaching values five to ten times the basal level. The kinetics of induction were essentially identical to those with tyrosine transaminase. Glucagon and isoproterenol, activators of hepatic adenyl cyclase, also induced PEPCK, whereas insulin and hydrocortisone were not active in this regard. Studies with isolated particulate fractions from fetal liver revealed a significant activation of adenyl cyclase by glucagon and isoproterenol. Cycloheximide blocked the increase in PEPCK activity, and cyclic AMP did not activate this enzyme in vitro. These results suggest that *de novo* synthesis of PEPCK was enhanced by cyclic AMP, presumably in the same manner as with tyrosine transaminase. Other liver enzymes such as hexokinase, glucokinase, glucose-6-phosphatase, pyruvate kinase, and glutamic-oxalacetic transaminase did not respond to similar exposures to cyclic AMP in the culture system. Coupled with the lack of stimulation of general protein or RNA synthesis, these data suggest that the effects of cyclic AMP on enzyme synthesis are selective.

In spite of its lack of effect on PEPCK when added alone, insulin substantially inhibited induction of this enzyme by cyclic AMP, isoproterenol, and glucagon. The fact that the action of cyclic AMP was blocked demonstrates that insulin must be acting antagonistically at a step beyond the synthesis of cyclic AMP, by either increasing the breakdown of the cyclic nucleotide or blocking its action directly. Studies on the stability of cyclic AMP in homogenates are currently being carried out to help decide between these alternatives.

In order to assess the physiological validity of these studies and to extend their scope, the ability of cyclic AMP to induce tyrosine transaminase, PEPCK, and possibly other enzymes in vivo was assessed. Tyrosine transaminase was readily induced by injections of the N^6,$O^{2'}$-dibutyryl analog of cyclic AMP (DBCAMP) into intact or adrenalectomized rats. As early as 20 to 30 min following injection of DBCAMP there was a twofold increase in transaminase activity. Activities five to ten times the basal level were reached within $1\frac{1}{2}$ to 2 hr following injection. Hydrocortisone, on the other hand, has only a slight effect on transaminase levels by this time. Using immunochemical labeling techniques it was found that DBCAMP markedly increased the *de novo* synthesis of the transaminase. PEPCK activity in vivo was also elevated by DBCAMP, some four- to sixfold within 4 hr. The response was detectable in adrenalectomized rats on a normal chow diet, but the effect was greatest when intact rats kept on a protein-free diet for five days were employed. Serine dehydrase also showed a substantial increase with DBCAMP, about threefold in 4 hr. Tryptophan pyrrolase, on the other hand, was not significantly affected by DBCAMP, although hydrocortisone readily induced this enzyme as well as the transaminase. In contrast, PEPCK and serine dehydrase were not significantly affected by the steroid. The selectivity of enzyme induction by these two agents is thus clearly underscored.

Presently we are trying to determine if other hepatic enzymes are regulated by cyclic AMP and by what means the cyclic nucleotide influences enzyme synthesis in a selective manner.

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25.3 HORMONAL CONTROL OF YOLK PROTEIN SYNTHESIS IN AMPHIBIAN LIVER: INDUCTION OF RNA SYNTHESIS BY ESTROGEN

J. L. Wittliff F. T. Kenney

We now have clear indications[1] that steroid hormones induce the synthesis of specific proteins in liver by acting as classical inducers, that is, by promoting mRNA synthesis. Direct evidence to support or reject this conclusion is lacking, however. Glucocorticoids promote a large and nonspecific burst in the labeling of RNA when given in vivo, which obscures the presumably selective effect of these hormones on mRNA synthesis.[2] In cultured cells no effect of glucocorticoids on RNA synthesis can be detected, in accord with the fact that synthesis of only one minor protein component is stimulated – that is, such a response would be expected to be virtually undetectable at the RNA level.

Accordingly, we have turned our attention to the induction by estrogens of yolk protein synthesis in the liver of male *Xenopus laevis*[3] as a change of sufficient magnitude that discernible alterations in RNA synthesis could be expected. As Wallace and co-workers have shown,[3,4] estradiol-17β stimulates a massive accumulation of these lipophosphoproteins in the serum of male toads, to a level comprising more than 50% of the total serum proteins. Further, in that these proteins apparently are absent in the serum of untreated males lies the promise that the steroid initiates the synthesis of a new species of RNA, if our considerations are correct.

Incorporation of isotopic RNA precursors (^{14}C-orotate or ^{32}P) into total liver RNA of male toads is markedly altered by estrogen treatment. Beginning between 3 and 9 hr after injection of estradiol-17β, the synthesis of RNA is increased threefold over the very low control rate. A 1-hr incorporation time serves as a "pulse" measurement of liver RNA synthesis. During this time the base composition of the pulse-labeled RNA, which is markedly DNA-like (A + U/G + C = 1.25), did not change appreciably. After 12 hr there was a second estrogen-dependent burst in RNA synthesis, leading to a rate five- to sixfold greater than the controls. This was associated with a shift in base composition toward the ribosomal type (A + U/G + C = 0.94). Pool sizes of the precursors in the liver appear to be unchanged by the hormone treatment.

To investigate further the nuclear response to estrogen, we attempted to isolate ribonucleoprotein particles similar to those which others[5] have shown to be the chromosomal product carrying messenger-type RNA in rat liver. Thus far we have not been able to identify these particles in *Xenopus* liver nuclei. However, we have shown, using continuous labeling experiments, that precursor incorporation into cytoplasmic 18 and 28S ribosomal RNA was increased twofold by the hormone 12 hr after hormone administration. An increase in labeling is also seen in the 4–8S region; this remains elevated as long as 24 hr following steroid treatment. We are presently attempting to segregate the various events in RNA synthesis following hormone treatment and hope to correlate these with the response on the level of yolk protein synthesis.

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25.4 HORMONAL CONTROL OF YOLK PROTEIN SYNTHESIS IN AMPHIBIAN LIVER: INDUCTION OF PROTEIN SYNTHESIS BY ESTROGEN

J. L. Wittliff F. T. Kenney

One of the major requirements of research in induction of protein synthesis is to show that the inducer actually elevates the synthesis of the protein in question. We are attempting to measure the specific rates of synthesis of the yolk proteins of *Xenopus laevis* (phosvitin and lipovitellin) as a function of estrogen treatment, using a sensitive radioimmunoprecipitin assay.[1] Such an assay requires a specific antiserum against the protein under investigation. We have recently prepared highly reactive rabbit antisera against lipovitellin purified from *Xenopus* oocytes but have been unsuccessful in preparing rabbit antisera against native phosvitin, presumably because of its highly phosphorylated nature. We are presently testing the antigenicity of partially dephosphorylated phosvitin. The antiserum against lipovitellin cross reacts readily with SLPP (serum lipophosphoprotein complex), which is thought to be a precursor of the yolk proteins.[2] The proteins have only been observed in the serum as a complex, and their independent synthesis by the liver has been

inferred from inhibitor studies and in vitro techniques.[2,3]

Using the specificity and sensitivity of the radioimmunoprecipitin assay, we are attempting to establish the following: (1) that the liver is the site of yolk protein synthesis, (2) the time course of the induction by estrogen, and (3) a correlation between specific protein synthesis (phosvitin and lipovitellin) and the synthesis of specific RNA species, presumably messenger RNA.

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25.5 HORMONAL REGULATION OF TRANSMISSION OF RNA FROM NUCLEUS TO CYTOPLASM

C. B. Hager L. E. Roberson
F. T. Kenney Joseph Kendrick

In previous reports we have suggested that the pancreatic hormones insulin and glucagon induce enzyme synthesis in the liver by altering the properties of the mechanism responsible for transmission of mRNA from its nuclear site of synthesis to the cytoplasmic readout system. This suggestion is based on the kinetics of response to these hormones, which are indicative of a mechanism allowing translation of an otherwise unreadable store of mRNA. As these inductions are blocked by actinomycin, the nuclear apparatus may be implicated. We are investigating this possibility using both in vivo and in vitro approaches.

We have attempted to measure the effect of these hormones on the rate of "chase" of labeled RNA from nucleus to cytoplasm in vivo. High-specific-activity ^{3}H-orotic acid was administered for short periods (15, 30, and 60 min) and then followed by cold orotic acid in at least 1000-fold excess. This procedure was ineffective in that labeling of nuclear RNA continued at the usual rate, regardless of the treatment with cold precursor. Apparently the extensive turnover of the rapidly labeled DNA-like RNA within the nucleus involves a nucleotide pool which is not readily equilibrated with subsequently added unlabeled precursor. Similar results were obtained in comparable experiments in isolated perfused rat livers. The proposed experiment cannot be done well unless a clear-cut "chase" is obtained, that is, unless labeling of nuclear RNA ceases before hormones are administered, because of the profound effects of these hormones on precursor pools. For these reasons we have temporarily shelved this approach.

Liver nuclei containing labeled RNA were prepared from rats given labeled orotic acid for short periods of time. These nuclei were incubated in vitro with liver cytoplasm from animals not given isotope, and the rate of passage of labeled RNA from nucleus to cytoplasm was determined under a variety of conditions. Supplementation with ATP and a generating system to maintain ATP doubled the rate of flow of RNA. Addition of several concentrations of insulin, glucagon, or cyclic AMP had no effect on this rate.

Although these experiments have been uniformly negative, we intend to continue study of the transmission process and its hormonal regulation. That two-thirds of the messenger-type RNA undergoes turnover within the nucleus and is thereby not translated provides a clear indication that this process must be carefully regulated.

25.6 ALTERED PROPERTIES OF RAT LIVER NUCLEI AFTER GLUCAGON TREATMENT

F. T. Kenney Joseph Kendrick

A now-classical procedure for preparation of nuclei from rat liver involves their separation on discontinuous sucrose gradients in which densities are adjusted to permit only nuclei to sediment to the bottom of the tube. In the course of analyses of hormonal effects on the "transmission" of RNA from nucleus to cytoplasm, it was found that glucagon treatment in vivo caused the hepatic nuclei to remain unsedimented by this procedure, apparently by reducing the density of the nuclei. Further investigation of this phenomenon revealed it to be correlated to the loss of glycogen which follows glucagon administration, and not obviously pertinent to the induction of enzyme synthesis. In rats subjected to a 48-hr fast and thus depleted of glycogen, the hormone is fully effective in stimulating enzyme synthesis but has no detectable effect on the hepatic nuclei, as these nuclei are not sedimentable from the glycogen-depleted livers whether the hormone is given or not. These studies suggest that the glycogen content of nuclei may be an important determinant of their density.

26. Microbiology

Electron Transport Mechanisms in Microorganisms

S. F. Carson[a]
M. I. Dolin

[a]Dual Assignments

26.1 MALIC-LACTIC TRANSHYDROGENASE

M. I. Dolin

Kinetic Studies. – Investigations concerning the mechanism of enzyme reactions are sometimes complicated by the fact that the substrates are involved in mobile equilibria. Examples of this phenomenon are the keto-enol tautomerism of keto acids and the hydration of aldehydes. From a practical point of view, it is necessary to know how these equilibria affect the observed kinetics of the reactions. In addition, an adequate description of the mechanism requires identification of the active form of the substrate.

A general approach to these problems has come from a study of the kinetics of malic-lactic transhydrogenase.[1] Oxalacetate, one of the substrates of the enzyme, exists in solution as an equilibrium mixture of the keto and enol forms. Under conditions used for kinetic studies of the transhydrogenase, the tautomerism of oxalacetate is catalyzed by the basic form of tris(hydroxymethyl)aminomethane (Tris). The experimentally determined tautomeric rate constants were found to be very small, in comparison with the kinetic constants of the enzyme. Rate equations were derived which showed that the time course for keto and enol oxalacetate appearance and disappearance, in the presence of enzyme, can be expressed accurately as a function of the tautomeric rate constants and the velocity of total oxalacetate formation or utilization.[2] These equations make it possible both to predict the conditions under which valid kinetic data can be obtained and to identify the enzymically active tautomer. Correspondence between experimental data and the theoretical rate equations has shown that the keto form of oxalacetate is the active form of the substrate for the forward and reverse directions of the reaction catalyzed by malic-lactic transhydrogenase.

The equations used in this work are perfectly general and can, in principle, be used to investigate other enzyme reactions in which two forms of the substrate are in equilibrium. Examples of such enzymes are (1) transaminases in which oxalacetate serves as the amino acceptor, (2) malic dehydrogenase, (3) various aldehyde oxidases and dehydrogenases.

Spectral Studies. – Titration of the transhydrogenase with either of the two keto acid substrates (pyruvate or oxalacetate) leads to partial bleaching of the 350-mμ band (the band attributable to bound DPNH) followed, at higher concentrations of keto acid, by formation of a new intermediate absorbing at 320 mμ. Simultaneous fluorimetric and spectrophotometric titration has made it possible to obtain more accurate values for the dissociation constant of the second of these two overlapping spectral changes. The dissociation constant for the formation of the 320-mμ intermediate formed with pyruvate ($K_D = 1 \times 10^{-3}$ *M*) and oxalacetate ($K_D = 1 \times 10^{-4}$ *M*) are in good agreement with the dissociation constants for the abortive complexes found in kinetic experiments. This lends support to the idea that the 320-mμ intermediates are the abortive complexes. These intermediates may be the carbonyl adducts of keto acid and enzyme-bound DPN.

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27. Plant Physiology and Photosynthesis

Plant Physiology (27.1–27.8)		Photosynthesis (27.9–27.11)	
A. H. Haber	Rhonda F. Irwin	W. A. Arnold	J. R. Azzi
D. E. Foard	Nina S. Hammer[c]	R. M. Pearlstein	
Patricia L. Walne[a]	L. L. Triplett	W. A. Gibson[a]	
D. H. O'Dell[b]	Helen J. Luippold[a]		
	Barbara E. Rothstein[d]		
	Paula J. Thompson[e]		

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[b]Research Participant
[c]Dual Assignments
[d]Temporary
[e]Student Trainee

27.1 ACTIONS OF GIBBERELLIC AND ABSCISIC ACIDS ON LETTUCE SEED GERMINATION WITHOUT ACTIONS ON NUCLEAR DNA SYNTHESIS

A. H. Haber Stella W. Perdue[1]
D. E. Foard Helen J. Luippold

Promotion and inhibition of DNA synthesis have been suggested as important causal events in the growth-promoting action of gibberellic acid (GA) and the growth-retarding action of abscisic acid (ABA) respectively.[2,3] We here report typical effects of GA and ABA during lettuce seed germination after seed irradiation that prevents detectable DNA synthesis as determined by two criteria: Feulgen microspectrophotometry and ^{3}H-thymidine incorporation into nuclear DNA.

Lettuce seeds were given 1.3 megarads of ^{60}Co gamma rays and then sown in petri dishes containing distilled water or solutions of GA or ABA. Whereas lower radiation doses are sufficient to prevent mitosis during germination, the 1.3-megarad dose was necessary to inhibit nuclear DNA synthesis, as will be shown in this report. After five days, germination of these irradiated seeds was 0% in water controls, 84% in 5×10^{-4} *M* GA, and 1.5% in 5×10^{-4} *M* GA + 100 ppm ABA. Thus germination of these seeds was very sensitive to GA and ABA.

By Feulgen microspectrophotometry the relative amount of DNA per nucleus was measured for individual cells from (1) the apical 0.5 mm of the radicle of unirradiated embryos before sowing and (2) the protruded 1.1 mm of five-day-old root tips from the irradiated seeds that had germinated in 5×10^{-4} *M* GA. After histologic examination we chose the 1.1-mm length in (2) because it contains the same number of cells as the 0.5-mm length in (1). Because there was no mitosis, any DNA synthesis during the five days after sowing the irradiated seeds in GA would have shown up as increases in DNA contents in (2) compared to (1). Each slide contained a squash of either the 0.5-mm apical portion of the radicle from an unsown seed (1) or the protruded 1.1-mm root tip from an irradiated seed after five days in GA (2). Each slide also contained a squash of a normal root tip (one to five days old) comprised of cells in all stages of mitosis; the telophase nuclei were used to determine the 2C nuclear DNA level, and the prophase and metaphase nuclei were used

to determine the 4C nuclear DNA level. In this way the DNA content of individual nuclei in unsown seeds (1) and in roots from irradiated seeds germinated in GA (2) could be directly measured relative to the normal 2C and 4C nuclear DNA levels on the same slides.

All of 300 nuclei examined in unsown embryos had a 2C DNA content. This finding is similar to that of Brunori and D'Amato,[4] who found only 2C nuclei in 14 of 15 unsown lettuce embryos; the 15th had some 4C nuclei. Feinbrun and Klein[5] found that in Grand Rapids lettuce seed germinated on ^{3}H-thymidine, all nuclei were labeled with the exception of a few epidermal nuclei; therefore it may be inferred that the vast majority of nuclei in their lettuce seed also had a 2C DNA content. In 300 nuclei examined from five-day-old seedlings from the irradiated, GA-treated seeds, all but four had a DNA level no greater than the 2C amount. This observation indicates that DNA synthesis was absent or negligible in the elongating portion of these seedlings during the first five days after sowing. The four 4C nuclei in the root tips from the seeds irradiated and then germinated in GA may actually have been initially present before irradiation and germination, because 4 of 300 vs 0 of 300 is not a statistically significant difference. Brunori and D'Amato[4] and Feinbrun and Klein[5] also suggest the presence of a few 4C nuclei in unsown lettuce embryos. Even if these four nuclei did undergo DNA doubling during germination of the irradiated grain in the GA solution, the amount of DNA synthesis is still rather small per embryo; moreover, some of these germinated seeds, all of which required GA for germination, are still entirely 2C and therefore could not have undergone any doubling of DNA in their cells.

Autoradiography was also used to verify absence of nuclear DNA synthesis in both seeds and seedlings in 5×10^{-4} *M* GA. Irradiated seeds and the seedlings in 5×10^{-4} *M* GA, when 0, 1, 2, 3, 4, 5, 6, and 7 days old, were exposed for 24 hr to a solution also containing ^{3}H-thymidine. Those seeds not yet germinated were cut through the widest part of the cotyledons to assure that the ^{3}H-thymidine reached the radicle. Unirradiated seeds and seedlings of the same age were also exposed for the same time to ^{3}H-thymidine solutions of the same concentration. By this criterion, also, no nuclear DNA synthesis occurred in seedlings from the irradiated, GA-treated seeds, because grains over nuclei were absent or negligible (i.e., no greater in number than over nearby cytoplasm). By contrast, unirradiated seeds and seedlings of the same ages showed heavy labeling of nuclei.

Since by the two different criteria there was little or no nuclear DNA synthesis during germination of the irradiated seeds in GA, the promotion of germination by GA here is unrelated to any effect on nuclear DNA synthesis. In these seeds germination involves and is measured by expansion of the very same cells shown here to lack DNA synthesis. The prevention of germination by ABA in the presence of GA cannot be attributed to prevention of nuclear DNA synthesis, since there was none to inhibit. Although we have no evidence for significant cytoplasmic DNA synthesis, our data cannot rule out the possibility of cytoplasmic DNA synthesis as clearly as nuclear DNA synthesis. Since all or nearly all the nuclei in the lettuce embryo are initially at the 2C DNA level, the normal mitotic divisions during germination of unirradiated seeds must involve DNA doublings. From the very great burst in mitotic frequency that typically accompanies the onset of normal germination, there is little question that, with nonirradiated seeds, GA and ABA would have exerted their expected effects on nuclear DNA synthesis associated with these mitoses. The results in the present communication show that such effects on nuclear DNA synthesis are not necessary for the actions of these chemicals on lettuce seed germination. A more detailed account of this work, including reconciliation of these results with studies[2] seeming to lead to an opposite conclusion, is in press.[6]

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27.2 RADIOSENSITIVITY AND RATE OF CELL DIVISION

A. H. Haber Barbara E. Rothstein

A generalization concerning the greater effectiveness of ionizing radiation on dividing compared with nondividing cells may have originated partly from early, subsequently confirmed, observations of irradiated tissues having different extents of mitotic activity.[1] These

tissues, however, differed in morphology and physiology as well as mitotic activity. More recent studies have focused on mitotic inhibition, chromosome breakage, and other irregularities of mitosis, which are detected with ease in dividing cells but either cannot occur or cannot be detected in nondividing cells. There have been no studies concerning radiosensitivity of dividing vs nondividing cells of the same morphological cell types when the biological criterion of damage or death is equally applicable to dividing and nondividing cells. A favorable test system for such a study is gamma-radiation-induced susceptibility to photodestruction of chloroplasts in tobacco leaf parenchyma. This system is favorable for two additional reasons: (1) the chlorenchyma grows with many cell divisions in young leaves, few in intermediate, and no cell divisions in older leaves;[2] and (2) the photodestruction is an early reflection of general cell lethality rather than of a specific destruction of chloroplasts[3,4] and is indicated by the decline in chlorophyll content in both dividing and nondividing cells. Even without ionizing irradiation, bleaching of green tissue resulting from chlorophyll destruction (in contrast to the occasional loss of capacity for plastid formation) in higher plants is a general characteristic of senescence.[5] Our system, unlike those mentioned above, permits comparison of doses necessary to produce the same biological effect in cells of the same morphological and physiological cell types.

Feulgen-stained leaf squashes indicated the expected presence of many mitotic figures in "young" leaves, 20 to 50 mm long; of fewer in "middle" leaves, 80 to 110 mm long; and of none in the "old" leaves, 130 to 155 mm long. The relative rates of increase in palisade cell numbers were 0.6 to 1.1 day^{-1}, 0 to 0.4 day^{-1}, and 0 respectively. Leaf disks 5 mm in diameter were punched from young, middle, and old leaves of the same tobacco plants, irradiated with ^{60}Co gamma rays, and floated under 450 ft-c of white light. Chlorophyll was analyzed after 24 hr illumination. The dose-response curves for gamma-radiation-induced photodestruction were similar, irrespective of the relative rate of cell division in the chlorenchyma. The chlorophyll loss represents gamma-ray-induced sensitivity to photodestruction, not direct destruction by gamma rays, because during 24-hr incubation in darkness there was negligible chlorophyll loss with or without gamma irradiation. After 1.1 megarads, which produced almost complete chlorophyll loss after one day in the light, the leaf disks lost chlorophyll slowly in darkness, but not faster than the unirradiated dark controls, which took about nine days to reach half their initial chlorophyll content. Thus the effect of gamma radiation here studied corresponds to one of the criteria of general lethality for photosynthetic tissue established in studies of wheat leaf chlorenchyma.[3]

These results do not contradict either the truism that many cytogenetic radiation effects are more easily observed in dividing than in nondividing cells or the generalization that after irradiation, cell divisions increase damage.[3,6] Within the same type of tissue, there undoubtedly are physiological differences associated with the presence or absence of cell divisions. Our results suggest, however, that such physiological differences in themselves do not significantly alter the intrinsic radiosensitivity of the whole cell. A more detailed account of this work is in press.[7]

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27.3 GAMMA-RADIATION-INDUCED STIMULATION OF LEAF GROWTH

A. H. Haber D. E. Foard L. L. Triplett

Low doses of ionizing radiations have been reported to promote plant growth.[1-3] To be detected, any stimulation of processes promoting growth must more than counteract the growth-retarding radiation effects of mitotic inhibition and of genic imbalance resulting from chromosome breakage in dividing cells. We investigated seedling irradiation effects in a system in which growth promotions, if present, can be more easily observed, because of the absence of the aforementioned two typical inhibitory radiation effects. Such a system is the gamma plantlet, a wheat seedling which grows without mitosis after 500 kilorads of gamma irradiation of the dry seed. Gamma plantlets have nuclear RNA synthesis, net protein synthesis, and differentiation

without any lethality or accelerated senescence.[4] Their growth requires soluble carbohydrate derived from breakdown of insoluble carbohydrate, principally starch, in the endosperm.[5]

Using four-day-old gamma plantlets of wheat of the Monon variety, we found that 25 kilorads of seedling irradiation promoted growth of the first and second foliage leaves but not the stem from which they arose. When the seedling is excised from the endosperm and grown on exogenous carbohydrate, there is no radiation-induced promotion of leaf growth. When the seedling is normally attached to the endosperm, the radiation-induced leaf growth promotion is accompanied by a greater breakdown of endosperm reserves to soluble carbohydrate. Intact gamma plantlets of variety Lemhi fail to show both the leaf growth promotion and the increase in soluble carbohydrate in the endosperm. Thus the promotions of leaf growth and of breakdown of insoluble carbohydrate seem to go together. In Monon endosperm the increase in soluble carbohydrate is not accompanied or preceded by changes in amylase activity. We suggest that the leaf growth promotion, not masked by mitotic inhibition and by gross genic imbalance, results from the more favorable carbohydrate nutrition caused by an apparently nonspecific promotion of endosperm breakdown. A preliminary account of this work has been published.[6]

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27.4 GROWTH STUDIES VALIDATING ANATOMIC STUDIES OF SHOOT RESPONSES TO SEEDLING IRRADIATION

D. E. Foard A. H. Haber

Previous observations of wheat initially irradiated as seedlings gave the expected results in roots, namely, cell lethality and collapsed meristems, presumably as the result of chromosomal imbalance due to chromosome breakage in dividing cells. The shoots of these seedlings, however, unexpectedly failed to show such cell lethality and meristem collapse at the same or higher doses. From anatomic observations alone we attributed the absence of cell lethality in the irradiated shoots to the apparent masking of chromosomal damage by the radiation-induced inhibition of cell division.[1] If this is true, seedling irradiation produces in the shoot a very similar effect to that observed in the entire seedling grown from seed irradiated sufficiently to prevent mitosis (the "gamma-plantlet"). For many cereals, one characteristic of gamma-plantlets is an increased growth of the leaf compared with that of seedlings grown from seed irradiated with slightly lower doses that do not prevent mitosis. In other words, there is a reversal, or "dip," in the curve relating leaf height to seed-irradiation dose.[2] In the leaf, growth and cell division are localized within the same group of cells; consequently, chromosomal imbalance due to chromosome breakage in dividing cells occurs in the same group of cells that causes the leaf to grow. Since there is no such localization of cell division and growth in the adjacent coleoptile, there is no dip in the curve relating coleoptile height to radiation dose.

The aforementioned responses of shoot structures to seed irradiation suggest a test of our conclusion concerning the shoot response to seedling irradiation. We irradiated normally growing seedlings with various doses of gamma rays and looked for a dip in the curve relating leaf height to radiation dose. The seedlings were excised from the endosperm and grown on sucrose plus nitrate to eliminate indirect effects on leaf growth resulting from irradiated endosperm.[3] We found a dip in the curve relating leaf height to radiation dose at about 25 kilorads. This dose is considerably less than the dose corresponding to the dip in the leaf height curve after seed irradiation but is consistent with our predictions from anatomic observations of the shoot after seedling irradiation. We conclude that for doses in excess of 25 to 50 kilorads, the absence of cell lethality in shoots results from the masking of chromosomal damage by the radiation-induced inhibition of cell division. This conclusion is supported further by the absence of a dip in the height vs dose curve in two types of irradiated seedling controls: (1) the coleoptiles of the same plants whose leaf growth did show a dip in the height vs dose curve, and (2) the leaf after seedling irradiation of gamma-plantlets (i.e., plants reirradiated as seedlings after already having received seed irradiation doses sufficient to prevent mitosis). Since the gamma-plantlet seedlings had no mitosis, they would

not be expected to show any seedling irradiation phenomena involving chromosomal imbalances due to chromosome breakage in dividing cells.

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27.5 COMBINED ANATOMIC AND AUTORADIOGRAPHIC STUDIES OF NUCLEIC ACID SYNTHESIS IN BARLEY ALEURONE

D. E. Foard Rhonda F. Irwin A. H. Haber

The two most intensively studied effects of the plant hormone gibberellic acid (GA) are on (1) growth promotions in a variety of plants and (2) promotion, in aleurone layers of barley endosperm, of de novo synthesis of hydrolytic enzymes: amylases,[1] proteases,[2] and perhaps ribonuclease.[1] In another report we demonstrated an experimental separation of GA action on growth from any action on nuclear DNA synthesis.[3] In this report we combine anatomic and autoradiographic studies of embryoless halves of GA-treated barley seeds. In this system GA has been shown to stimulate synthesis of the hydrolytic enzymes despite absence of overall growth in aleurone cells.

We used Himalayan barley from the same source as the Himalayan barley in which GA stimulates the synthesis of the hydrolytic enzymes. Using experimental techniques and sampling times employed in the enzyme synthesis studies,[1] we included ^{3}H-thymidine in the incubation mixture for embryoless halves of the grain. After incubation for one to six days in the mixture containing GA and ^{3}H-thymidine, the endosperm halves were fixed, serially sectioned, and prepared for autoradiography. By this criterion no nuclear DNA synthesis occurred in the aleurone of GA-treated endosperm halves, because grains over nuclei were absent or negligible.

This result does not exclude the possibility, among others, that the labeled precursor did not penetrate the cells of the aleurone. Two observations suggest that this possibility is unlikely: (1) parallel experiments with ^{3}H-uridine demonstrate incorporation of the precursor into insoluble RNA in nuclei and cytoplasm of aleurone cells, and (2) parallel experiments with ^{3}H-thymidine in embryo-containing halves demonstrate incorporation of the precursor into DNA by the embryo, but not by the aleurone, even though part of the aleurone overlies the immediately adjacent embryo. On the basis of these experiments we infer that the action of GA on enzyme synthesis in barley aleurone proceeds without action on nuclear DNA synthesis. This conclusion concerning GA action on enzyme synthesis in the nongrowing system thus parallels our conclusion concerning GA action on growth itself.[3] The barley aleurone, which has not been studied by combined anatomic and autoradiographic means, may become a useful system for studies of nuclear and cytoplasmic RNA synthesis associated with enzyme synthesis but uncoupled from overall cell growth, cell division, and nuclear DNA synthesis. Also, in contrast to our other studies,[3] no treatment such as large doses of radiation is required.

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27.6 EFFECT OF LIGHT ON ULTRASTRUCTURE OF SENESCING CHLOROPLASTS AND AN APPROACH TO CORRELATING CHLOROPHYLL CONTENT PER PLASTID WITH STAGE OF DISINTEGRATION

Patricia L. Walne A. H. Haber L. L. Triplett

Light controls the green status of the plant not only by permitting chlorophyll synthesis but also by retarding chlorophyll destruction.[1] We here report the ultrastructure of senescing chloroplasts in 3-cm apical wheat leaf tips excised and floated on 2×10^{-4} *M* aminotriazole (to prevent chlorophyll synthesis and chloroplast formation) and 10^{-5} *M* DCMU (to prevent functionally complete photosynthesis). After four days of either continuous darkness or 6-hr daily photoperiods of 900 μw/cm^2 white light, the leaf sections, together with zero-time controls, were fixed for electron microscopy and analyzed for chlorophyll. We found three stages that, although arbitrary, were convenient for classifying the ultrastructure of chloroplasts in a senescing population. These classes are:

1. *Intact* – chloroplast envelope intact and compressed; grana and stroma lamellae compact, without waviness, and largely oriented parallel to the long axis of the plastid; matrix dense and nonvacuolate.

2. *"Partly disintegrated"* – chloroplast envelope slightly separated and vacuolate in places but otherwise intact; grana and stroma lamellae swollen and separated, with incipient to severe reorientation toward the periphery of the plastid; matrix less dense than in (1).

3. *"Greatly disintegrated"* – chloroplast envelope ruptured; grana and stroma lamellae oriented toward periphery of plastid and highly vacuolate; matrix less dense than in (2), and there is considerable increase in size and number of osmophilic granules in both the matrix and interlamellar regions.

For the leaf tissue incubated in darkness a total of 725 chloroplasts (in 100 cells) were assigned among the categories as follows: intact, 29%; "partly disintegrated," 47%; "greatly disintegrated," 24%. For the corresponding tissue incubated with illumination, a total of 218 chloroplasts (among 30 cells) were assigned as follows: intact, 78%; "partly disintegrated," 21%; "greatly disintegrated," 1%. We conclude from the highly significant difference that light controls the disintegration of mature chloroplasts in the absence of functionally complete photosynthesis. A preliminary account of this portion of the problem has been published.[2]

From the chlorophyll contents per leaf section in zero-time controls (c_0 = 28.5 μg), after dark incubation (c_D = 10.9 μg), and after light incubation (c_L = 23.1 μg), we can calculate a very rough estimate of the relative chlorophyll content of individual plastids in the three ultrastructural categories described in the preceding paragraph. Let x, y, and z represent the chlorophyll content of an intact, a "partly disintegrated," and a "greatly disintegrated" chloroplast respectively. Let N be the total number of chloroplasts of all categories per leaf section; since there is no chloroplast formation, N should have the same value for all treatments. The following three simultaneous equations relate plastid chlorophyll to total chlorophyll in the zero-time controls, dark-incubated, and light-incubated tissue respectively. The coefficients of x, y, and z represent the fraction of the total number of chloroplasts in the corresponding categories.

$$Nx = c_0 \,, \tag{1}$$

$$N(0.290x + 0.472y + 0.239z) = c_D \,, \tag{2}$$

$$N(0.780x + 0.211y + 0.009z) = c_L \,. \tag{3}$$

From these equations, $y = 0.14x$ and $z = 0.10x$. Consequently, the ultrastructural stages here described as "partly disintegrated" and as "greatly disintegrated" contain very roughly 14 and 10% of the chlorophyll of an intact mature chloroplast. The accuracy of this method of calculation depends not only upon the statistics of sampling but also upon several biological assumptions that remain to be critically examined.

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27.7 CRUDE ACTION SPECTRUM FOR RETARDATION OF CHLOROPLAST SENESCENCE

Paula J. Thompson A. H. Haber

In the absence of chlorophyll synthesis and chloroplast formation, the loss of ultrastructural integrity of mature chloroplasts can be followed as a decline in chlorophyll content.[1] In this study we examine such chlorophyll loss under equal intensities of continuous illumination with light of various colors. Apical 3-cm leaf sections were excised from eight-day-old wheat seedlings and floated on solutions of aminotriazole (to prevent chlorophyll synthesis and chloroplast formation) in petri dishes. The dishes were put in light-tight boxes covered with Corning narrow-band-pass filters of ten different colors. For each individual filter, even including those supposedly identical, we determined the transmission spectrum in a Cary spectrophotometer with the kind help of M. I. Dolin of the Microbiology Group. Light from a bank of incandescent lights passed through a Plexiglas trough with running water (to remove infrared radiation and heat) and individual neutral density filters, each adjoining one of the glass color filters. For each sample of leaf sections the transmission of the neutral density filter was adjusted so that the total incident light intensity reaching each sample of tissue was the same.

The results, in the crude action spectrum in Fig. 27.7.1, show maximal effectiveness of blue and red light and minimal effectiveness of green and near-infrared radiation. Owing to the temperature rise with increasing illumination over the large area for each sample of leaf sections, we were unable to get sufficiently high intensities to determine a true action spectrum (i.e., a curve in which the ordinate represents the reciprocal of light intensity necessary to produce a given effect). Nevertheless, the shape of the curve is

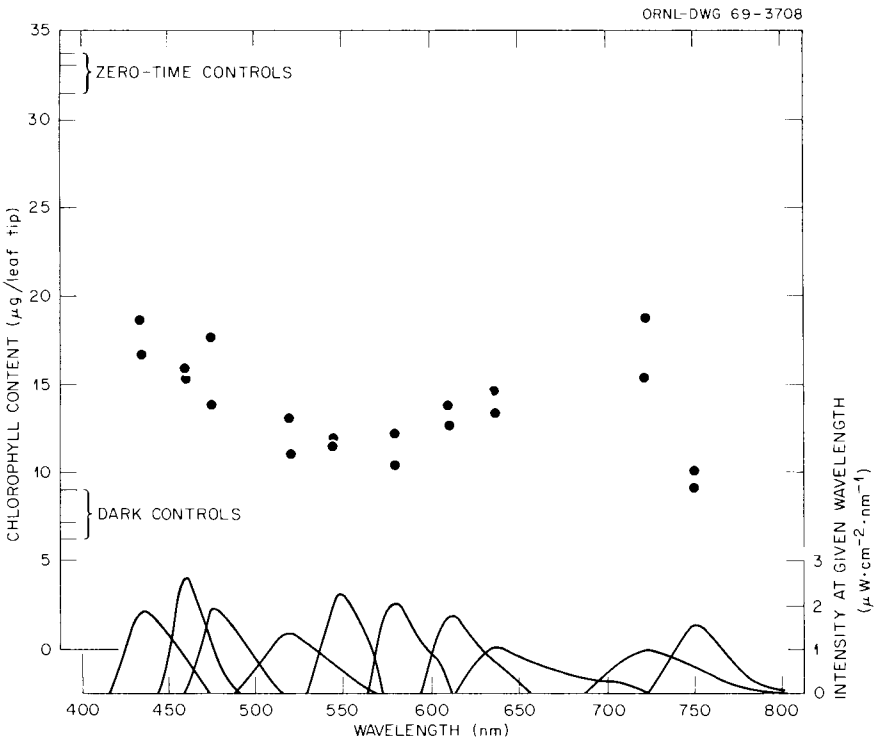

Fig. 27.7.1. Effect of Light Quality in Preventing Chlorophyll Decay. The leaf tips were floated on 10^{-3} *M* potassium phosphate buffer (pH 6.1) and 2×10^{-4} *M* aminotriazole at 31° for three days. All illuminated tissue continuously received illumination at an intensity of 57 $\mu w/cm^2$, the area under each of the ten curves at the bottom of the figure; the spectral distribution of each color is given by the shape of these curves. For the illuminated leaf tips the chlorophyll contents are plotted at the wavelength of maximum intensity.

sufficiently different from crude action spectra of functionally complete photosynthesis to suggest that the light effect here studied is not mediated by functionally complete photosynthesis. This suggestion is consistent with the ineffectiveness of DCMU in preventing the light effect.[1] The crude action spectrum in Fig. 27.7.1 is remarkably similar to the crude action spectrum for photomorphogenesis mediated in several systems by the "high energy reaction."[2]

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27.8 LATERAL ROOT PRIMORDIA IN THE RADICLE OF BUCKWHEAT EMBRYOS

D. H. O'Dell D. E. Foard Rhonda G. Irwin

Although adventitious root primordia may occur in mature embryos of certain species, for example, *Zea mays*, there are no reports known to us of genuine lateral (branch) root primordia within parent root primordia in seeds. We here report the occurrence of true lateral root primordia in the radicle of the mature embryo of ungerminated buckwheat (*Fagopyrum sagittatum* Gilib.) achenes.

Longitudinal sections of the ungerminated embryo reveal that protuberances of the pericycle occur at intervals along the length of the pericycle within the radicle-hypocotyl axis. Each protuberance is mostly biseriate, whereas regions of the pericycle between protuberances are only uniseriate. In the vicinity of the protuberances, the endodermis and one or more files of cortical cells are displaced outward. Pericyclic protuberances occur as close as 130 μ from the apex of the radicle proper (i.e., exclusive of the radicle cap). There are approximately 20 protuberances per radicle in this batch of buckwheat.

Transverse sections of the ungerminated embryo reveal that the pericycle protuberances occur opposite xylem ridges and between successive phloem ridges in

this tetrarch root. Thus the origin of the protuberances is identical to that of lateral root primordia in typical tetrarch dicotyledonous roots, that is, in the pericycle opposite xylem ridges.[1] Thus these protuberances possess a definitive characteristic of genuine lateral root primordia, namely, correct site of origin.

Sections of growing seedlings reveal that these protuberances also possess another definitive characteristic of lateral root primordia, the capacity to develop into lateral roots. In seven-day-old seedlings the number of emerged lateral roots is approximately equal to the number of protuberances in the embryo. We conclude, therefore, that these pericyclic protuberances are genuine lateral root primordia, which were formed during embryogeny. So far as we are aware, this is the first report of such a phenomenon. A detailed description of this work is in press.[2]

We have also observed lateral root primordia within very short lateral roots of buckwheat seedlings. The anatomy of these primordia is similar to that of primordia in the radicle of the embryo. In contrast to the condition in the vast majority of plants, these primordia are formed very close to the tip of the growing parent root. This exceptional tendency of buckwheat to form lateral root primordia makes it a favorable species to use for studies of organogenesis.

References

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27.9 PHOTOSYNTHESIS

W. A. Arnold J. R. Azzi J. B. Davidson[1]

Photosynthesis, the photoreduction of carbon dioxide by green plants, is the only major process by which energy is brought into the biological cycle. The work of Calvin, Benson, and co-workers[2] has elucidated the enzyme chemistry by which carbon dioxide is converted to sugars. However, the initial light-gathering steps are still unclear. By what mechanism is a plant able to convert the energy of light quanta into chemical oxidizing and reducing power which is available for utilization in the biochemical reduction of carbon dioxide to sugars? More specifically, how does the absorption of light by chlorophyll lead to the production of those high-energy chemical substrates, adenosine triphosphate and reduced pyridine nucleotide, which initiate the dark reactions of the carbon cycle? We are studying the delayed light and fluorescence to learn about the initial steps.

Energy Storage in Photosynthetic Organisms at Low Temperature. – Frozen green plants are able to store light energy, as is shown by the reemission of light on heating. We have determined the activation energies for three of the peaks of emission that we see on heating the sample from −196 to +100°C.

We have published these measurements, together with a discussion of their possible connection with the energy levels of chlorophyll that R. C. Nelson has determined.[3,4]

We have made determinations of the optical cross section for two of the peaks in the glow curve. The cross sections are not the same; they differ by 1.5X. They correspond to several hundred chlorophyll molecules.

Tris-Treated Chloroplasts. – Butler and Yamashita[5] have shown that by treating chloroplasts with 0.8 *M* Tris it is possible to have a sample which cannot produce oxygen but still can transfer electrons through system II.

We have made some observations on this system and find that the glow curves are very small, but that delayed light is very strong at short times. This suggests that Tris-treated chloroplasts have an electrical "short" between the two sides.

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27.10 DECAY PROFILE OF BETA-PARTICLE-EXCITED FLUORESCENCE OF CHLOROPHYLL IN GREEN PLANTS

R. M. Pearlstein G. B. Price, Jr.[1]
W. A. Gibson

Work is continuing on the development of an instrument to determine the fluorescence decay profile of

chlorophyll in biolamellar structures (such as chloroplasts) and of other biomolecular systems. In the instrument, beta particles from a ^{90}Sr source impinge on a thin sample. Beta particles with sufficient energy to penetrate the sample are subsequently detected by a plastic scintillator and photomultiplier. Photons, emitted by molecules excited by beta particles, which occasionally emerge from the sample, are detected by a second photomultiplier. The distribution of time intervals between pairs of events detected by the two photomultipliers is recorded on a multichannel analyzer. The tail of this distribution yields the desired decay profile of a fluorescent sample. If a thin piece of plastic (Cerenkov radiator) is used as a test sample, the distribution is a symmetric curve whose full width at half maximum provides a measure of the instrumental resolving time. Last year we reported a resolving time of 700 psec (0.7 nsec); this time has been reduced to 330 psec. Quite recently, the photomultiplier electronics has been modified in a way that gives a resolving time of 240 psec (occasionally even less) in a gamma-gamma coincidence experiment used as a calibration test. We hope to incorporate this improvement into the beta-particle instrument.

In its present form the instrument is suitable for detection of fluorescence having a wavelength of 600 nm or less. We have, for example, observed with it the fluorescence of phycoerythrin in the red alga *porphyridium*. Some further work may be required to improve the signal-noise ratio at wavelengths as long (680 to 720 nm) as those emitted by chlorophyll *a*.

The Neutron Physics Division has continued to be extremely cooperative. We acknowledge particularly the work of N. Hill, who is on loan to that Division from the Instrumentation and Controls Division.

Reference

[1] Student at the UT–Oak Ridge Graduate School of Biomedical Sciences.

27.11 EXCITON TRAPPING IN PHOTOSYNTHETIC UNITS AND RELATED SYSTEMS

R. M. Pearlstein

Theoretical studies of the exciton trapping problem begun in the previous year are continuing. The most interesting new result to emerge from these studies in 1968 concerns the effect of exciton trapping in linear polymers on the fluorescence kinetics of those polymers. In 1967, I reported[1] that strong energy transfer coupling between monomers can lead to a slower rate of trapping in a linear polymer than weak coupling. Specifically, if either (or both) of the end monomers of the polymer is a trap, and there are N monomers in the polymer, the rate of trapping of the longest-lived strong-coupling mode is proportional to N^{-3}, that for the corresponding weak-coupling mode to N^{-2}. The proportionality constant is such that for $N > 8$ the weak rate is faster. As long as the trapping rate is much larger than the fluorescence rate of a similar N-monomer polymer *without* a trap, the lifetime of the longest-lived component of fluorescence in the polymer *with* a trap is proportional to N^3 in the strong case and to N^2 in the weak. This result appeared to provide a basis for distinguishing strong from weak coupling in polymers by experimentally determining fluorescence yield as a function of polymer length (in monomer units), since fluorescence yield is often proportional to fluorescence lifetime. Now I have found that in the contemplated experimental situation this proportionality between yield and lifetime does not even hold approximately in the presence of strong coupling. In fact, the theoretical fluorescence yield is proportional to N^2 for *both* strong and weak coupling, and with the *same* proportionality constant in the two cases. Thus the N^2 and N^3 dependences could only be distinguished in experiments which resolve the shape of the fluorescence decay curve sufficiently to determine the lifetime of the longest-lived fluorescent component directly from that curve. The instrument described in the preceding section already has such a capability for polypeptides and polynucleotides.

Reference

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28. Microbial Photosynthesis

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28.1 PTERIDINES IN PHOTOSYNTHETIC REDUCTION

N. A. Nugent R. C. Fuller

Previous evidence from this and other laboratories has suggested that the nonconjugated pteridines may play a role in photochemical electron transport.[1,2]

It has been postulated that the primary photoact in photosynthesis produces a strong reductant with a redox potential of around −600 to −700 mv. The two most important properties of a candidate for this primary reductant are: (1) the candidate must be photoreducible by a photosynthetic system and (2) since ferredoxin is photoreduced, any primary reductant should be capable of reducing ferredoxin. The possibility that a pteridine functions in this capacity was the subject of this investigation. Specifically, the ability of a reduced pteridine to reduce ferredoxin was determined.

The reduction of ferredoxin was carried out by measuring the difference in the absorption spectrum of the oxidized and reduced forms. 2-Amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine reduced spinach ferredoxin according to the equation given below:

pteridine H_4 + ox-ferredoxin
$\longrightarrow$ pteridine H_2 + red.-ferredoxin

The reduction was virtually instantaneous and the equilibrium far toward reduced ferredoxin. Tetrahydropteridine is thus able to replace the photoreductant in photosynthesis as far as the reduction of ferredoxin is concerned.

Thus it is clear that the pteridines have a lower chemical potential than ferredoxin and are consequently closer to the potential produced by the primary photochemical act.

References

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28.2 PHOTOSYNTHESIS IN *RHODOSPIRILLUM RUBRUM*. IV. ISOLATION AND CHARACTERIZATION OF RIBULOSE-1,5-DIPHOSPHATE CARBOXYLASE

Louise E. Anderson R. C. Fuller

Ribulose-1,5-diP carboxylase [3-phosphate-D-glycerate carboxylyase (dimerizing), EC 4.1.1.39] catalyzes the formation of 2 moles of 3-P-glyceric acid from 1 mole of CO_2 and 1 mole of ribulose-1,5-diP. This reaction is ubiquitously distributed among autotrophic organisms, including the photosynthetic and chemosynthetic bacteria, blue-green algae, and higher and

Table 28.2.1. Purification of Ribulose-1,5-diP Carboxylase from *R. rubrum*

Step	Protein (mg)	Units[a]	Specific Activity[b]	Purification	Recovery (%)
225,000 × *g* supernatant	130	38	0.31		
$MnCl_2$; $(NH_4)_2SO_4$ fractionation	57	51	0.9	2.9	>100
G-200 Sephadex	14	17	1.2	3.9	44
Hydroxylapatite	0.47	2.3	4.8	15	5.8

[a]Micromoles of 3-P-glyceric acid formed per minute.
[b]Micromoles of 3-P-glyceric acid formed per minute per milligram of protein.

lower plants.[1-4] In higher plants the carboxylase is a high-molecular-weight protein (mol. wt 557,000, $s_{20,w}$21) that makes up as much as 16% of the soluble protein of the green leaf.[5] Although the specific activity of the enzyme in extracts of photoautotrophically grown *Rhodospirillum rubrum* is higher than in green plant extracts,[6] the *s* value of the carboxylase is only 6.2, indicating a much smaller protein.[7] It is immediately evident that these two carboxylases are quite different in molecular structure and possibly in activity. It was therefore of interest to isolate and characterize the carboxylase from this photosynthetic bacterium and to compare the smaller ribulose-1,5-diP carboxylase of *R. rubrum* with the carboxylase of higher plants.

Ribulose-1,5-diphosphate carboxylase has been isolated from autotrophically cultured *Rhodospirillum rubrum*. The molecular weight is 120,000. The K_m for ribulose-1,5-diphosphate is 83 m*M* and for CO_2 is 59 m*M*. The enzyme is inhibited by three important metabolites: (1) citrate, an intermediate of the tricarboxylic acid cycle; (2) inorganic phosphate; and (3) 3-phosphoglyceric acid, the product of the reaction catalyzed by the carboxylase. The kinetic properties of these inhibitions are given in Table 28.2.1. Both the levels and the activity of ribulose-1,5-diphosphate carboxylase are apparently subject to metabolic control in this facultative photoautotroph.

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28.3 EFFECTS OF CHLORAMPHENICOL AND CYCLOHEXIMIDE ON AMINO-ACID-INCORPORATING SYSTEMS FROM THE CHLOROPLAST AND CYTOPLASM OF PLANTS

R. M. Smillie Nicholas Rigopoulos
Bonnie J. Reger R. C. Fuller

Introduction. – Considerable knowledge has been acquired on the role of chloroplast DNA and chloroplast ribosomes in the biogenesis of the chloroplast. Smillie has employed the inhibitors chloramphenicol (CAP) and cycloheximide and *Euglena* in in vivo experiments and has found: (1) Calvin cycle enzymes and electron-transfer-pathway proteins are synthesized within the chloroplast and on chloroplast ribosomes (70*s*), and (2) chloroplast DNA codes for chloroplast ribosomal RNA. Chloramphenicol apparently inhibits 70*s* ribosomes, whereas cycloheximide inhibits 80*s* ribosomes. We have now obtained in vitro concentration curves for the effect of CAP and CH on ribosomal systems from chloroplasts and cytoplasm of *Euglena*, wheat, and pea. Such data were obtained for dark- and light-grown plants.

Results. – The amino-acid-incorporating systems of wheat, pea, and *Euglena* chloroplasts were almost totally dependent on ATP and Mg^{2+}, to a lesser extent on GTP and amino acids, and appeared not to be stimulated by ammonium ions. Because whole chloroplasts were used, strict attention was paid to bacteria and other organelle contaminates. Platings of reaction mixtures were done routinely, counts of chloroplasts to

mitochondria were made, and activities were determined.

The experiments with wheat proplastids and chloroplasts have been essentially completed. The following table consists of data pertaining to wheat:

Counts per Minute per Milligram of RNA

		μg/ml CAP						
	Control	5	10	20	40	80	200	800
Proplastids	664	366	286	180	148	118	86	
Chloroplasts	704	490	351	286	220	198	147	104

		μg/ml CH			
	Control	1	5	20	200
Proplastids	556	425	556	525	406
Chloroplasts	632	618	593	561	561

The results indicate: (1) proplastids of wheat must be very well developed, since there was no significant increase in activity between the dark- and light-grown plants; (2) chloramphenicol drastically reduced incorporation, although even at 800 μg/ml activity was not zero; and (3) cycloheximide (CH) at 200 μg/ml affected activity by only 11 to 27%. The effect of these inhibitors on cytoplasmic ribosomes remains to be investigated.

Experiments on pea and *Euglena* are in progress. Indications so far are that the data on inhibitors will be much the same; however, one difference is considerably increased activity with green chloroplasts vs proplastids. The cytoplasmic ribosomes of pea and *Euglena* will also be studied.

29. Biophysics

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29.1 ISOLATION AND CHARACTERIZATION OF CRUSTACEAN RIBOSOMAL RNA

Dorothy M. Skinner

Methods for the isolation of ribosomal RNA (rRNA) from three crustacean tissues have been devised. These include the removal of both melanin and ATP by precipitation of rRNA from total RNA preparations with 1 *M* sodium chloride or solubilization in buffer containing EDTA to chelate Mg^{2+} and Ca^{2+} which, when present, form alcohol-insoluble complexes with ATP. By cosedimentation or gel electrophoresis with rRNA of known $S_{20,w}$, the sedimentation constants of crab rRNA have been determined to be 28*S* and 18*S*. The base compositions of the 28*S* and 18*S* rRNA's determined are similar to those of other metazoans. Muscle, midgut gland, and epidermis have been found to have higher concentrations of rRNA in the premolt than in the intermolt period, with the maxima corresponding to the periods of maximum protein synthesis. The highest concentration of rRNA is found in premolt epidermis when it is synthesizing the new exoskeleton.[1]

Reference

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29.2 LOCALIZATION OF THE CISTRONS FOR RIBOSOMAL RNA IN A CRUSTACEAN

Dorothy M. Skinner

Three percent of the DNA isolated from gonads of the land crab *Gecarcinus lateralis* is comprised of a (G + C)-rich satellite that has a base composition similar, if not identical, to the DNA which codes for ribosomal RNA in other species.[1,2] Although, as shown by saturation experiments, about 2.5% of the genome of *Gecarcinus* is specific for rRNA, the hybridization does not occur with the (G + C)-rich satellite, but rather with DNA slightly denser (ρ = 1.704 g/cm^3) than the peak of the main-band DNA (ρ = 1.701 g/cm^3).[3]

RNA isolated from a 100,000 × *g* pellet of a homogenate of cultured *Xenopus* liver cells labeled for 8 hr and chased for 35 hr or from purified HeLa cell (labeled for 24 hr and chased for 18 hr) ribosomes associates with crab DNA of the same density (ρ = 1.704 g/cm^3). It seems likely therefore that the RNA bound is authentic rRNA.

The large fraction of DNA associating with rRNA suggests that the ribosomal cistrons are present in multiple copies. The association of rRNA with a sheared DNA of density only slightly greater than the average main-band density rather than with a DNA of a density corresponding to a G + C content of rRNA (ρ = 1.722 g/cm^3) suggests that the cistrons for rRNA are not clustered. An alternate explanation for these data is that the rDNA is comprised of unusual bases such that the d(G + C) content cannot be calculated from the CsCl buoyant density; there is no evidence for this alternative.

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29.3 STUDIES ON THE TIME RELATIONSHIPS BETWEEN DNA SYNTHESIS AND THE MITOTIC CRISIS IN THE EPIDERMIS OF THE LAND CRAB *GECARCINUS LATERALIS*

Catherine Tchernigovtzeff Dorothy M. Skinner

Although crabs increase in size at each molt, their constituent cells do not. This observation implies that mitosis must occur in conjunction with ecdysis; nevertheless, cell division has not previously been observed in this species. By radioautographic studies of ^{3}H-thymidine-injected animals, we find in epidermal cells (1) negligible DNA synthesis and no mitosis during intermolt; (2) DNA synthesis during D_0, the earliest premolt stage; and (3) a burst of mitotic activity in stage D_1. (Stage D_1 is also the stage in which the epidermis resorbs 75% of the old exoskeleton; in stage D_2 it synthesizes a new exoskeletal replacement.)[1] Mitoses are not observed again until the next premolt period. We estimate that no more than 5% of all epidermal cells divide at any one molt; whether these same cells or a different population divide at the subsequent molt is not known.

The microscopic observations were complicated by the fact that in this animal the amount of uric acid present is so great that sheets of fixed epidermal tissue were usually opaque. However, the tissue could be cleared and the nuclei easily visualized if the tissue were shaken overnight in a solution of 5% sodium carbonate, pH 11. By this simple procedure, essentially all of the uric acid is removed from the tissue.

Reference

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29.4 CHROMOSOME REPLICATION IN *ESCHERICHIA COLI*

L. G. Caro Claire M. Berg[1]
D. P. Allison

Our purpose was to locate the origin of chromosome replication on the circular genetic map of a number of isogenic strains of *E. coli* K12 of different mating types and to determine the direction of replication. In various experiments several criteria were used to define the origin: (1) gradient of marker frequency in exponential cultures growing in minimal medium, (2) probability of position of growing point in exponential cultures, (3) probability of position of growing point in cultures starved for an essential amino acid and in cultures in which the amino acid had been restored, (4) marker frequency gradient in cultures growing in rich and poor media, and (5) marker frequency gradient in cultures shifted from a poor to a rich medium.

Neither of the parameters used, marker frequency or growing point position, can be determined absolutely. Measurements can only be relative, comparing one condition to another or one culture to another; hence the necessity for using as many criteria as feasible. The method for assay for marker frequency was generalized

transduction by the phage Pl. That for position of growing point was labeling with bromouracil, followed by infection with Pl, separation of transducing particles containing bromouracil DNA on a cesium chloride gradient, and assay of transducing activity.

The results showed: (1) that in several isogenic F^-, F^+, and F' strains (derived from *E. coli* K12 W1485) the origin of replication is located between the markers *arg*G and *xyl* on the genetic map; (2) that in these strains the direction of replication is clockwise; (3) that in Hfr strains of the same family the replication pattern during amino acid starvation is altered: the origin appears on a point diametrically opposed to the one previously defined and the direction is counterclockwise; it is not fully established whether these conclusions are valid for the normal replication pattern or reflect only the behavior of the replication point during amino acid starvation; (4) that all Hfr strains in the group studied have the same replication pattern regardless of the site or the direction of integration of F and that F does not therefore seem to be the origin of replication.

Reference

[1] University of Connecticut, Storrs.

29.5 STUDY OF TRANSDUCTION BY PHAGE Pl

Grete Kellenberger-Gujer

Phage Pl is able to cut out genetic material from the chromosome of the host bacterium in which it multiplies, to wrap this host DNA up in Pl protein coats, and thus is able to transduce almost any known bacterial marker at low frequency from one cell to another. It has been shown by Ikeda and Tomizawa[1] that the particles in a Pl lysate which transduce bacterial markers contain mostly (and probably only) bacterial DNA. These authors used 5BU-labeled ("heavy") donor cells for their experiments. We confirmed their findings for donor cells made heavy either with ^{13}C or ^{15}N or with deuterium. Under our conditions of labeling, no partial degradation and reutilization of the bacterial DNA for Pl formation is observed. Therefore we could answer the question as to whether Pl does or does not distinguish between its own DNA pool and the bacterial DNA once maturation and encapsulation starts. Differential label experiments show that, regardless of what proportion of the DNA appears in mature particles (20 to 50%), about six times more of the Pl DNA than of the bacterial DNA is encapsulated. The discrimination between bacterial and phage DNA thus is relatively poor. Part, if not all, of the preference given to phage DNA could be due to the fact that the protein pool is located closer to the phage DNA pool than to the bacterial nucleus. Preliminary morphological studies (L. Caro, personal communication) indicate that, unlike T4-infected cells, the bacterial nucleus is not dissolved after Pl infection and furthermore that particle formation occurs in clearly defined regions of the cell, which may be situated adjacent to, but not mingled with, the bacterial nucleus.

The physical uptake of bacterial DNA is unexpectedly high in comparison with the low transduction frequency measured genetically. One can calculate from our results that about 1 out of 20 particles of a Pl lysate is a transducing particle. The chance that a given selected marker is contained in it is about 1:30. Thus about 1 of 600 Pl particles should contain the selected marker, but successful transduction is found only at a frequency of 1 to 2×10^{-4} under optimum conditions. Therefore nine out of ten transducing particles would lead to abortive transduction. For some markers, abortive transduction can be scored for, and the results fit the expectation, namely, that ten times more abortive than normal transductants are found. The reason why a piece of DNA introduced into the bacterial cell and functioning as an informative unit can no longer be incorporated and multiplied with the bacterial chromosome is presently under study.

Reference

[1] H. Ikeda and J. I. Tomizawa, *J. Mol. Biol.* **14**, 85 (1965).

29.6 GENETIC RECOMBINATION AT THE MOLECULAR LEVEL

Grete Kellenberger-Gujer R. A. Weisberg

The chromosome of bacteriophage λ consists of a single molecule of double-stranded DNA comprising about 50,000 nucleotide pairs. It had been previously found that genetic recombination in λ is accompanied by material exchange of DNA and occurs by breakage and reunion of the parental chromosomes.[1,2] Since then several recombination systems for λ have been discovered: the *Int* system, in which recombination at a specific site is promoted by a λ gene product; the *Red* system, which is also under the control of a λ gene product; and the *Rec* system, directed by bacterial genes. *Red* and *Rec* are not site specific. Since the material exchange of DNA in recombination has been so far observed only for all the three recombination

systems working together, the question arises whether this observation holds separately for each of them. We therefore repeated some of the earlier experiments under conditions in which only one of the recombination genes was allowed to express itself, the others being inactivated by mutations. The transfer of density-labeled parental DNA to recombinants was measured. Qualitative results indicate that recombinations promoted by *Int* as well as *Red* or *Rec* occur by breakage and reunion of the parental genomes. However, we discovered that in the absence of an active *red* gene in the cross, there was extensive degradation and reutilization of the parental (labeled) phage DNA. This made any precise quantitative measurements of recombinational exchange difficult. We are presently trying to find out why, in the absence of any recombinant formation (all minus system) or in the presence of only the bacterial recombination system (low recombinant yield), this high level of turnover is observed.

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29.7 EXPRESSION OF THE *N* GENE AND STABILITY OF ITS PRODUCT IN λ LYSOGENS

R. A. Weisberg

The *N* gene of phage λ seems to be required for the full expression of most other λ genes. The expression of gene *N* itself is under the direct control of the λ repressor, since it cannot be induced without inactivation of the repressor, and the *N*-gene product is apparently required only in catalytic amounts for normal phage growth. In strains lysogenic for a λ mutant which synthesizes a reversibly thermolabile repressor (λ857), an extremely brief heat pulse will permit normal growth of a heteroimmune superinfecting N^- phage. Thus gene *N* is "fully" expressed very soon after repressor inactivation. However, if the briefly heated λ lysogen is allowed to grow at low temperature (33°C), it rapidly loses its ability to support the growth of a heteroimmune N^- phage. The functional half-life of the *N*-gene product, measured in this way, is about 5 to 10 min. In contrast, the activity of the *N*-gene product is quite stable in briefly heated cells which are stored in an ice bath.

29.8 THE INTEGRATION AND EXCISION DEFECT OF PHAGE λ*dg*[1]

R. A. Weisberg M. E. Gottesman[2]

Transducing phage λ*dg* differs from bacteriophage λ in the low frequency with which it integrates into and excises from the bacterial chromosome. Nevertheless, on infection or induction, λ*dg* can produce the diffusible elements required for integrative recombination, since it complements and is complemented by the mutant λ*int6*. *Cis-trans* tests of the ability of various phages and prophages to complement the integration-excision defect of λ*dg* have shown that such complementation can only occur in *cis*. These observations lead us to conclude that λ*dg* lacks a structural element needed for normal integration and excision.

We have accounted for this defect by assuming that the attachment region of the λ chromosome is not completely homologous to the bacterial attachment region. Thus *Int*-promoted recombination between the phage and bacterial chromosomes at the attachment regions will result in a prophage which is bordered at its left- and right-hand termini by two new and structurally unique prophage attachment regions. The frequency of *Int*-promoted recombination between different attachment regions must depend on the structure of the pair involved, with recombination between λ*dg* (which carries the left prophage attachment region) and the bacterial chromosome evidently being infrequent. Although the molecular basis of this dependence is unknown, we can show that the recombination frequency is not a simple function of the amount of homology shared by the two recombining structures.

References

[1] This report is a continuation of: R. A. Weisberg, *Biol. Div. Ann. Progr. Rept. Dec. 31, 1967,* ORNL-4240, p. 290.

[2] Laboratory of Molecular Biology, National Institute of Arthritis and Metabolic Diseases, Bethesda, Md.

29.9 STUDIES ON THE PENETRATION MECHANISM OF THE BACTERIOPHAGE f1

Ann B. Jacobson Dorothea V. Parker

The bacteriophage f1 is a filamentous DNA-containing virus which only infects cells of *Escherichia coli* carrying the sex factor F. Cells which carry the F factor have filamentous appendages known as F pili to which

the phages attach. The mechanism by which phage DNA penetrates into the bacterial cell following attachment is not known, but it presumably involves the F pilus.

We are studying the process of DNA penetration into the bacterial cell by high-resolution electron-microscope autoradiography. Preliminary experiments have been done to define the timing of the penetration process more closely. These studies are designed to show the length of time the phage DNA remains associated with the F pilus after penetration has begun. F1 phages labeled with ^{3}H-thymine were attached to cells at 0°C. Unattached phages were washed free by filtration, and the remaining bacteria-phage complexes were incubated at 37° to allow DNA penetration to occur. At various time intervals, samples were taken and pili removed from the cells by a brief ultrasonic treatment. The labeled DNA which remained with the cells following the ultrasonic treatment was measured. These experiments showed that all phage DNA penetration occurs within 15 min after incubation at 37° has begun. The penetration of f1 DNA can be stopped by attaching the male specific RNA phage MS2 or by placing the cells in NaCN shortly after DNA penetration has been initiated.

Radioautographs are prepared with whole bacteria to which f1 phages have been attached. The cells are fixed after varying amounts of phage DNA have penetrated into the cells. Then the bacteria-phage complexes are placed on grids, shadowed with Au-Pd, and coated with emulsion. The radioautographs are developed after one to two months of exposure. The first radioautographs prepared this way have been difficult to interpret, because phage attachment has been variable and the techniques for preserving the cells have also been inadequate. Recently we have been able to improve both the phage attachment conditions and conditions for cell fixation. Radioautographs are currently in preparation using these improved procedures.

29.10 X-RAY BREAKDOWN OF THE DNA OF *HAEMOPHILUS INFLUENZAE* FOLLOWED BY CELLULAR DEGRADATION AND REPAIR

M. L. Randolph Jane K. Setlow[1]

Introduction. – Study of x-ray-induced breakdown of the DNA of a series of ultraviolet-sensitive mutants of *Haemophilus influenzae* and competition between subsequent cellular degradation and repair of the DNA complements and extends previous studies on similar ultraviolet-induced lesions.[2] This report describes experimental results and gives at least qualitative interpretations of such studies.

Results and Discussion. – Log-phase cells, their DNA labeled with radioactive thymidine and suspended in growth medium, were given x-ray (250 kvp, HVL 0.5 mm Cu, h = 0.3) exposures of 0 to 60 kr and molecular weight distributions were determined from measurements of radioactivity distribution after ultracentrifugation in alkaline sucrose gradients. Each gradient contained irradiated cells labeled with ^{3}H and, as a control, unirradiated cells labeled with ^{14}C. Weight-average molecular weights were computed using a computer program based on the triple isotope program of V. A. Singletary and J. E. Donnellan, Jr.[3] Results (Table 29.10.1) of calculations of the number of breaks per strand based on these averages, cell survival measurements,[2] and target theory calculations for the direct effect of x rays on a strand of molecular weight 4 × 10^{8} daltons are given in Table 29.10.1. There are, on the average, about 1.5 single-strand breaks per lethal event for the Rd and DB 116 mutants, one single-strand break per lethal event for the x-ray-sensitive mutant DB 117, and 1.5 to 2 primary ionizations (produced by the direct effect of x rays) per single-strand break.

In other experiments, tritium-labeled cells were incubated for various durations in growth medium at 37°C after irradiation, following which the total acid-insoluble tritium was measured or the molecular weight distribution determined. Semilogarithmic plots of the breakdown of DNA to acid-soluble bits vs incubation duration are initially steep but plateau within 2 hr at proportionately higher values than cell survival. The distribution of molecular weights shifts toward the normal distribution with increasing incubation time. Although these changes are caused partly by more rapid enzymatic degradation of the low- than the high-molecular-weight pieces of DNA, various analytic tests of the data suggest that repair of broken single strands

Table 29.10.1. Effects of X-Ray Irradiation in Vivo of the DNA of *Haemophilus influenzae*

Phenomenon	Dose Required (kilorads)
One primary ionization per single strand (direct effect)	1.5
One break per single strand	2.8
D_{37} for DB 117 mutant	2.5
D_{37} for Rd and DB 116 mutant	4.0

also occurs. This return to normalcy is quicker in Rd cells than in DB 116.

Irradiation of purified transforming DNA (which imparts to these cells resistance to cathomycin and streptomycin) in buffer and in a protective medium (10% yeast extract) indicated that the indirect effect of x rays is at least 100 times the direct effect. Transforming DNA, when irradiated in the protective medium but not in buffer, is more radiation sensitive if assayed on DB 116 cells than on Rd. Thus resistance of transforming DNA to the direct effect of x rays seems directly proportional to how fast recipient cells rejoin single-strand breaks in their DNA. These data suggest that (1) directly induced single-strand breaks in transforming DNA are more likely to be repaired than lesions produced by indirect induction and (2) the repair rate is less important for transforming DNA than for cell survival, perhaps because complete enzymatic degradation of cellular DNA strands probably takes much longer than for the shorter strands of transforming DNA.

Measurements of the synthesis of acid-insoluble DNA following irradiation by incubation in growth medium containing tritium-labeled thymidine indicate that the cells have little or no pool of thymidine and that the total synthesis after a period equivalent to five cell division times is roughly proportional to cell survival.

References

[1] Cytology and Genetics section.

[2] J. K. Setlow *et al., J. Bacteriol.* **95**, 546 (1968).

[3] V. A. Singletary and J. E. Donnellan, Jr., "A Computer Program for Some Biological Problems," this report, paper 29.25.

29.11 PHOTOREACTIVATION AND PHOTOREACTIVATING-ENZYME ACTIVITY IN AN ORDER OF MAMMALS

J. S. Cook James D. Regan[1]
Susumu Takeda

Although photoreactivating-enzyme activity has been found in all phyla so far examined, neither the enzyme nor the phenomenon of direct photoreactivation has heretofore been demonstrated in the class Mammalia. By several criteria, we now find photoreactivation readily demonstrable among marsupials, even among species of widespread geographic origin. Tissues examined include two established lines of cultured cells from the Tasmanian rat kangaroo, a line from the South American woolly opossum, and six primary tissues from locally trapped specimens of the opossum *Didelphis marsupialis*. Ultraviolet-irradiated kangaroo cells show weak photoreactivation of growth. Extracts made from any of the nine tissues show strong photoreactivating-enzyme activity in their ability (1) to restore, in the light, transforming activity to uv-irradiated transforming DNA from *Haemophilus influenzae* or (2) to monomerize thymine-containing photodimers in uv-irradiated *E. coli* DNA. Despite the ability of such extracts to repair heterologous DNA, we find no similar activity on the part of irradiated kangaroo or woolly opossum cells to repair their own DNA in vivo. This discrepancy enhances the probability that the enzyme performs some function in metazoa other than photoreactivation of uv lesions. The reason for the discrepancy is currently being investigated.

Reference

[1] Mammalian Cytogenetics section.

29.12 AN ENDONUCLEASE THAT ACTS ON ULTRAVIOLET-IRRADIATED DNA's

W. L. Carrier R. B. Setlow

Extracts of *M. lysodeikticus* contain nuclease activities that excise dimers from uv-irradiated DNA without extensive degradation of the DNA. One of the nucleases involved in excision is an endonuclease that is specific for uv-damaged DNA. The use of a simple, convenient endonuclease assay employing small Sepharose columns[1] has permitted us to purify the endonuclease and determine a number of its properties.

The endonuclease makes single-strand breaks in native uv-irradiated DNA. It is inactive on unirradiated DNA (native or denatured). The numbers of chain breaks resulting from enzymic treatment increase with increasing uv dose to the DNA. At 4×10^4 ergs/mm^2 (280 nm) the average chain length is reduced to ~100 nucleotides – a value close to the average distance between the cyclobutane pyrimidine dimers that uv makes in the DNA. If DNA irradiated in aqueous solution is subjected to enzymic photoreactivation (to monomerize the cyclobutane dimers), subsequent endonuclease action makes a negligible number of chain breaks. These data indicate that the dimer-containing regions in native DNA are the substrate.

We have determined that the chain break appears in the strand carrying the dimer (and not in the region opposite the distortion produced by the dimer) in the following way. Unirradiated ^{32}P-labeled DNA was annealed with an excess of irradiated ^{3}H-labeled DNA.

The annealed molecules were treated with endonuclease. Only the ^3H strand was affected.

Irradiated poly dA·dT is 100-fold better as a competitor than is irradiated poly rA·rU, and irradiated RNA's do not compete for the enzyme. DNA that contains the "spore-type" photoproduct (made by irradiation at low temperatures) does compete with DNA that contains cyclobutane dimers. The sedimentation constant of the enzyme is similar to that of cytochrome c, indicating that it is a low-molecular-weight protein, and its absorption spectrum shows that it has low or zero amounts of tyrosine and tryptophan.

Reference

[1] R. B. Setlow and W. L. Carrier, *Biol. Div. Ann. Progr. Rept. Dec. 31, 1967,* ORNL-4240, p. 297.

29.13 THE BIOLOGICAL EFFECT OF A PURIFIED ENDONUCLEASE SPECIFIC FOR DAMAGED DNA

R. B. Setlow Jane K. Setlow[1]
W. L. Carrier

After transforming DNA from *Haemophilus influenzae* has been exposed to ultraviolet radiation outside the bacteria, the survival of transforming ability varies with the strain used as a recipient cell. Wild-type cells are better able to repair uv damage in transforming DNA than a uv-sensitive mutant.[2] When a purified endonuclease from *Micrococcus lysodeikticus* is incubated with uv-irradiated DNA from *H. influenzae*, the survival of transforming ability assayed on the mutant increases to that observed normally on the wild type. The enzyme must therefore provide a function that is defective in the mutant, which is unable to excise pyrimidine dimers from its DNA.

The enzyme is assayed chemically by its ability to make single-strand nicks in uv-irradiated DNA. It does not affect the transforming ability of unirradiated DNA and can therefore be assumed to be free of nonspecific DNase. However, when uv-irradiated DNA is treated with the enzyme and then assayed on wild-type recipients, the transforming activity decreases with time of enzyme treatment. We have obtained evidence that (1) the single-strand nicking, (2) the reactivation of transforming ability observed with mutant cells, and (3) the decrease in transforming ability observed with wild-type cells are one and the same activity. The three activities vary the same way with column fractionation, pH, heat inactivation, inhibition by cyanide, and phosphate concentration. The inactivation of enzyme-treated DNA that is observed when it is assayed on wild-type cells may be interpreted to mean that the wild-type cells, which normally can make single-strand nicks in the first step of repair of pyrimidine dimer damage, must carry out the subsequent steps in some temporal or spatial pattern which follows the first step. This pattern is disrupted when the single-strand nicks are made outside the cell.

It is concluded that (1) the enzyme as isolated from *M. lysodeikticus* is almost certainly part of a repair system and (2) the uv-sensitive mutant lacks the ability to make single-strand nicks as the first step in repair of uv damage.

References

[1] Cytology and Genetics section.
[2] This report, paper 1.5.

29.14 PYRIMIDINE DIMER EXCISION AND HOST-CELL REACTIVATION OF PHAGE λ

J. M. Boyle R. B. Setlow

The survival of many phages following ultraviolet (uv) irradiation is dependent on the bacterial strain used in the survival array. Higher survivals are obtained on bacteria (Hcr^+) which are said to host-cell reactivate the uv-induced lesions than on Hcr^- bacteria, which are not able to perform this function. When bacteria are irradiated their inactivation is correlated with the formation, in deoxyribonucleic acid (DNA), of cyclobutane-type pyrimidine dimers. The higher survival of Hcr^+ strains is correlated with the excision of these dimers and subsequent repair of the DNA. Thus it seemed likely that host-cell reactivation of phages would also be correlated with excision of pyrimidine dimers from the phage DNA by the host's excision enzymes. However, attempts to demonstrate such a correlation with phages T1 and T7 have been unsuccessful.[1]

The present system employs λ, a temperate phage of *E. coli.* We have shown that the production of both thymine-thymine and cytosine-thymine dimers in the DNA of uv-irradiated λ phage particles has the same dose dependence as *E. coli* DNA. Further, 55 to 70% of all dimers are selectively excised from uv-irradiated λ DNA during 100 min of incubation at 37° by λ-Hcr^+ complexes, whereas during the same period, only 13% of dimers are removed in λ-Hcr^- complexes. In this experiment λ-induced protein synthesis is inhibited by 50 μg/ml chloramphenicol throughout the 100-min incubation period. The pattern of dimer excision from λ DNA in Hcr^+ and Hcr^- complexes correlates with the removal of uv-induced delay of intracellular phage synthesis by Hcr^+ complexes and with failure to remove

this delay in Hcr$^-$ complexes. These experiments confirm the results of Roulland-Dussoix,[2] who showed, using ^{32}P labeling, that there is more degradation of uv-irradiated λ DNA following incubation in Hcr$^+$ than in Hcr$^-$ strains.

Experiments are in progress which attempt to demonstrate: (1) a molecular basis for uv reactivation of uv-irradiated λ and (2) that phages which survive uv irradiation have had dimers excised and do not represent a fraction of the phage population which have not received potentially lethal hits.

References

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[2] D. Roulland-Dussoix, *Mutation Res.* 4, 241 (1967).

29.15 REPAIR OF SPORE-TYPE PHOTOPRODUCTS IN VEGETATIVE CELLS

J. E. Donnellan, Jr. R. S. Stafford

Ultraviolet irradiation of bacterial spores produces thymine photoproducts which are chromatographically different from the cyclobutane-type pyrimidine dimers produced in vegetative cells or in free DNA. The repair of those photoproducts in germinating spores also appears to occur by a mechanism different from that found in vegetative cells. The excision repair in vegetative cells results in the conservation of dimers in that all the dimers are recovered either in the growth medium or in the acid-soluble portion of the cell. In germinating spores, on the other hand, the spore photoproducts are not conserved but rather disappear from the DNA, presumably by reversion to thymine. The spore-type photoproducts are also formed in DNA and in cells irradiated in the frozen state. In fact, at −196°C the spore-type photoproducts make up more than half the thymine photoproducts. We are investigating the repair of photoproducts in vegetative cells following their irradiation at −196°C.

Vegetative cells of *Micrococcus radiodurans, Escherichia coli,* and *Bacillus megaterium* have been irradiated at −196°C and room temperature and then allowed to repair at 37°C in nutrient medium. The first two organisms are not spore formers, but the strains used possessed efficient mechanisms for the excision of cyclobutane-type dimers from their DNA following irradiation at room temperature. Spores of *B. megaterium* exhibited the nonconservative repair of the spore-type photoproduct, while vegetative cells excised cyclobutane dimers in the normal conservative manner, again following room-temperature irradiation.

After irradiation at −196°C and subsequent incubation in nutrient medium, both types of thymine photoproduct were excised conservatively from the acid-insoluble fraction (DNA) of cells of *M. radiodurans* and *E. coli.* The spore-type photoproduct was rapidly excreted into the medium by both these organisms, whereas much less cyclobutane dimer was found in the medium following repair by *E. coli.*

Although *M. radiodurans* normally excretes the cyclobutane dimer into the medium, this photoproduct is normally retained within the *E. coli* cell (acid-soluble fraction) for some time following irradiation at room temperature. Since the dimer was retained by *E. coli* following low-temperature irradiation while the spore-type photoproduct was excreted, we suggest the two photoproducts are treated differently by these cells. In addition, repair of the spore-type photoproduct in cells appears different from its repair in spores, since we observed a conservative repair in these experiments. It will be of interest to see if the cell of the spore former, *B. megaterium,* retains any of the nonconservative repair mechanisms of its spores or whether it repairs the spore photoproduct by a completely conservative mechanism.

29.16 ULTRAVIOLET RADIATION DAMAGE TO BACTERIAL CELLS AND THE REGULATION OF CELL METABOLISM

P. A. Swenson R. L. Schenley
Barbara A. Hamkalo

The rates of respiration and growth in *Escherichia coli* cells after uv irradiation depend on the ability of the cells to repair the damage to their DNA and on the composition of the media in which the cells are grown, irradiated, and incubated after uv. In strain B_{s-1}, unable to repair the radiation damage, growth quickly ceases but respiration continues for hours. Strain B/r can repair uv damage, and under certain conditions respiration is severely inhibited for a time after uv. Except with very high doses, respiration and growth are closely coupled processes at all times. With glycerol as a carbon source in synthetic medium supplemented with casamino acids, respiration continues for a time after uv and is then severely inhibited for a dose-dependent period of time (e.g., about 2 hr with a dose of 500 ergs/mm^2). All experiments described below are with this dose. If the cells are grown with or without casamino acids and irradiated and incubated in the absence of casamino acids, the inhibition is almost complete and lasts for about 4 hr. If casamino acids are added after uv, the period of inhibition is shortened,

the time decreasing with the increasing concentrations of casamino acids.

If cells are grown on glucose plus casamino acids and irradiated in the same medium, the respiration rate is slightly reduced but no severe temporary inhibition results. If, however, the cells are washed free of glucose, irradiated, and then given glucose, a severe temporary inhibition similar to that of glycerol results. When cells are grown on glycerol, irradiated, and switched to glucose, they behave like glycerol-grown cells. If unirradiated cells are switched to glucose for various lengths of time before irradiation, the period of inhibition becomes shorter; but, even if the cells divide three times in glucose, an inhibitory period characteristic of glycerol-grown cells occurs. Thus, by varying conditions, a wide range of respiration responses can be obtained; however, we have detected no significant differences in survival after irradiation under the various conditions.

We consider the severe temporary inhibition of respiration in irradiated cells as a means by which the normal ratios of DNA to RNA and protein are established in preparation for cell division. Because the effect is photoreactivable and is seen only in cells with repair mechanisms, the inhibition is thought to be through a metabolic control system activated by uv-damaged DNA.

We have begun a search for the site of inhibition of respiration in completely inhibited cells. The block is not in the cytochromes of the electron transport system, as indicated by difference spectroscopy of irradiated cells under anaerobic and aerobic conditions. The terminal oxidase spectra of irradiated and unirradiated cells poisoned with carbon monoxide are identical, and NADH oxidase levels are unchanged. In the Krebs cycle the succinic dehydrogenase level of irradiated cells is about 80% of that of the unirradiated cells.

Pulsation of both types of cells with uniformly labeled glycerol gives chromatographic patterns of cell extracts that are quite different. Most of the radioactive spots for the unirradiated cells are missing from the chromatograms of the extracts of irradiated cells. These differences are being investigated.

29.17 ATP LEVELS IN UV-IRRADIATED *ESCHERICHIA COLI* B/R AFTER PHOTOREACTIVATING TREATMENT

Barbara A. Hamkalo P. A. Swenson

Ultraviolet irradiation of *E. coli* B/r grown on glycerol as a carbon source results in the accumulation of 2.5 times the amount of cellular ATP that is present in unirradiated cultures. The high levels of ATP are measurable 10 min after uv, shortly before a severe temporary inhibition of respiration sets in. A high concentration of ATP can inhibit the Krebs cycle;[1] if the inhibition of respiration results from the accumulation of ATP, photoreactivation treatment which prevents this inhibition should result in a decrease in the cellular ATP concentrations.

Twenty minutes of black light treatment (300 to 400 nm, with a peak at 60 nm) at 37°C prevents inhibition of respiration and reduces ATP per cell to early control values. Black light treatment at room temperature prevents severe temporary respiratory inhibition but does not permit a significant reduction in cellular ATP. The reduction of the high levels of ATP in irradiated cells by photoreactivating treatment at 37°C seems to result from the increased utilization of ATP by actively metabolizing cells during the PR treatment. The absence of a reduction in ATP levels after room-temperature PR and the prevention of respiratory inhibition under these conditions indicate that the accumulation of ATP after uv does not cause inhibition of respiration.

Reference

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29.18 THE ACTION SPECTRUM OF THE INHIBITION OF THE INDUCED FORMATION OF TRYPTOPHANASE FORMATION IN *ESCHERICHIA COLI* B/r CELLS BY NEAR-ULTRAVIOLET RADIATIONS

P. A. Swenson R. B. Setlow

Near-ultraviolet (uv) radiation from a black lamp (peak output at 365 nm) has a strong but temporary inhibitory effect on the induced formation of tryptophanase in *E. coli* B/r. The action spectrum for this inhibition has a peak at 334 nm and is very similar to that for photoprotection and growth delay in *E. coli* B. A dose of 1.2×10^5 ergs/mm^2 at 334 nm inhibits respiration and growth by about 50% for 50 to 60 min, after which these processes proceed at near-normal rates. The same dose inhibits the induced synthesis of tryptophanase by about 95% for 40 to 50 min, and slow recovery follows. The close coupling between growth and respiration after near-uv radiation indicates that the primary effect of the photons is on some respiratory chain component such as quinones. However, the great sensitivity of the tryptophanase synthe-

sizing system is not easily explained by a reduced rate of respiration because the induced synthesis of β-galactosidase is relatively insensitive to near-uv radiation.

29.19 THE EFFECT OF CONFORMATION ON THE PHOTOCHEMICAL HYDRATION OF CYTIDINE

R. B. Setlow W. L. Carrier

Ultraviolet irradiation of the nucleotides of uracil and cytosine results in the addition of water across the 5,6 double bond. Such hydrates in messenger RNA's change the coding properties of the RNA, and some people think that cytidine hydrates in DNA can explain some of the mutagenic effects of uv radiation. The formation of cytidine hydrates may be detected by the lability of the ^{3}H atom in cytidine-5-^{3}H.[1] The DNA of *E. coli* is labeled with ^{3}H-cytidine and, as a control, with ^{14}C-cytidine. After uv irradiation the sample is heated, and the amount of ^{3}H appearing in the surrounding aqueous medium is separated from DNA by chromatography on Sephadex, charcoal, or DEAE.

The quantum yield for hydrate formation is independent of wavelength and dose for dCMP and for denatured DNA. The yield for irradiated native DNA is about tenfold smaller than for denatured DNA, and it tends to increase at high doses. A simple interpretation is that large doses result in the formation of locally denatured regions surrounding pyrimidine dimers and in these regions hydrate formation proceeds at a rate typical of denatured DNA. Typical values are shown below.

	Numbers of Photoproducts Formed per Absorbed Photon
Hydration of dCMP	6×10^{-3}
Hydration of denatured DNA	0.7×10^{-3}
Hydration of native DNA	0.07×10^{-3}
Dimers in native DNA	2×10^{-3}

Reference

[1] L. Grossman, *Photochem. Photobiol.* 7, 727 (1968).

29.20 PHOTOPRODUCT FORMATION IN FILMS OF DNA: DEPENDENCE UPON RELATIVE HUMIDITY

R. O. Rahn J. L. Hosszu

Irradiation of a dry DNA film results in both thymine dimer and spore photoproduct formation.[1] Because of our current interest in the nature of the conditions which favor the formation of the spore photoproduct, we studied the variation in photoproduct production in films of DNA equilibrated at various relative humidities. Very little, if any, spore photoproduct was formed in films equilibrated at relative humidities above 65%. At these high relative humidities the yield of dimer for a saturating dose of uv was the same as that obtained for DNA in solution. A sharp increase in spore photoproduct occurred as the relative humidity was deceased below 65%. Simultaneously the yield of thymine dimer decreased by a factor of 2. We conclude that these changes in the photochemistry reflect the known conformational change in DNA films from the B form to a disordered form as the water content is decreased. This conformational change, as observed by other workers[2] who measured optical properties such as absorbance or circular dichroism, occurs between 55 and 75% relative humidity. The region in which the changes in photochemistry occur is shifted slightly to lower humidities. The reason for this shift is not known at present. The characteristic absorption-desorption hysteresis previously observed[2] for the variation in optical properties with relative humidity was also observed for the photochemical changes.

We conclude that the sharp increase in spore photoproduct below 65% relative humidity is a result of the loss of the DNA B conformation. However, simply denaturing DNA, as by heating, is not sufficient to allow spore photoproduct formation.[3] It appears that a reduction in the water content is also important. Such a situation may exist in spores.

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29.21 STRUCTURAL CHANGES ACCOMPANYING UV IRRADIATION OF DNA

R. O. Rahn L. C. Landry

Irradiation of DNA in solution leads to a lowering and broadening of the thermal melting profile.[1] This effect is presumed to be due to the formation of photoproducts, which lead to a weakening of the forces stabilizing the double-strand helix. Of interest are the photochemical events mainly responsible for this effect. Setlow and Carrier[2] have obtained evidence that thymine dimers may be only in part responsible.

To study this question, we have irradiated DNA in the presence of ethylene glycol, a denaturant which lowers the melting temperature. Irradiation at 25°C of native DNA in 90% ethylene glycol results in nearly complete denaturation of the DNA at this temperature. As expected, if the glycol only serves to lower the melting temperature, increasing the salt concentration, lowering the glycol concentration, or lowering the temperature at which the absorbance measurement is made prevents uv denaturation. Hence irradiation and subsequent absorbance measurement at 25° of DNA in the presence of glycol eliminates the need to run melting curves at elevated temperatures in order to determine the effect of uv on the lowering of the melting temperature. Propylene glycol was found to be a more effective denaturant than ethylene glycol for this purpose.

The uv-induced absorbance changes in the presence of other denaturing agents such as copper and formaldehyde were also investigated. These agents, in addition to lowering the melting temperature, react specifically with the bases following denaturation. However, irradiation in the presence of these reagents resulted in absorbance changes accounted for by the effects of these agents solely on the melting temperature; that is, no additional denaturation was observed due to specific interactions with the uv-denatured regions.

The formation of uv-induced cross-links prevents the separation of strands following irradiation and denaturation. Of current interest is the question of whether the photoreactivating enzyme can repair these cross-links. Hydroxylapatite columns are being used to distinguish between single- and double-stranded DNA following denaturation with base. We have evidence that DNA which has been irradiated and then denatured contains portions which behave on the column in a way intermediate between native and denatured DNA. We hope to further use hydroxylapatite column chromatography to help distinguish between the different structural states of DNA which arise following uv irradiation.

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29.22 PHOTOCHEMICAL STUDIES OF THYMINE IN ICE

R. O. Rahn J. L. Hosszu

Irradiation of thymine in ice produces, in addition to the thymine dimer, two other photoproducts. One of these photoproducts is the thymine-thymine adduct, which absorbs with a maximum at 315 nm due to a pyrimidine-one ring.[1] We have determined that the maximum yield of this photoproduct for irradiation at −196°C is about 3.2%. Upon additional irradiation at −196° this photoproduct decreases, and another, a proposed "trimer," is formed with a maximum yield of 10%. The trimer shows a sigmoid dose-response curve, which signifies that a uv-induced precursor is needed for its formation. Since irradiation of the trimer in solution leads to the adduct plus thymine, a possible precursor for the trimer could be the oxetane derivative proposed[1] as an intermediate in the formation of the adduct. Fluorescence measurements at −196°C show that the adduct is not formed directly at −196°C but comes about upon annealing at temperatures greater than −80°.

Absorption measurements of thymine ice films at −196°C show that uv irradiation leads to an appreciable absorbance decrease at −196°. In particular, there is a complete loss of the structured and red-shifted crystalline absorption spectrum. This structured spectrum presumably arises from strong exciton interactions between the bases. Irradiation either selectively removes (via dimerization) those bases which are responsible for the structured spectrum or else dimerization leads to isolated thymines which do not have neighbors to interact with.

We propose then that upon irradiation of thymine in ice at −196°C (1) dimers are formed directly as judged by the large absorbance decrease; (2) an oxetane, or some similar precursor, is formed which interacts with another thymine to form a trimer; and (3) upon annealing, the above precursor is converted to the adduct.

Reference

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29.23 ELECTRON SPIN RESONANCE STUDIES OF THE TRIPLET STATES OF MONOANIONIC THYMINE

R. O. Rahn

A solution of the monoanion of thymine (pH > 11) consists of an equilibrium mixture of two tautomeric forms which correspond to the loss of a proton at either the N_1 or N_3 position.[1] These two forms have distinctly different absorption spectra, and changes in the equilibrium distribution are easily followed

spectrophotometrically. The addition of ethylene glycol favors loss of the N_1 proton, while lowering the temperature favors loss of the N_3 proton. It is not possible to resolve the phosphorescence emission from the two different tautomers because of spectral overlap.[2] However, electron spin resonance measurements of thymine (pH 12) at $-196^{\circ}C$ revealed two clearly resolved uv-induced $\Delta m = \pm 2$ triplet signals corresponding to the two possible tautomers of the monoanion. The variation of these two signals with the excitation wavelength compared favorably with the absorption spectra of the two tautomers. Hence electron spin resonance is capable of distinguishing between the two different tautomer triplet states of monoanionic thymine.

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29.24 LABELING OF BASES AND PHOTOPRODUCTS IN UV-IRRADIATED DNA WITH ^{3}H-DIMETHYL SULFATE

J. S. Cook

In order to study ultraviolet (uv) damage to DNA and its repair in cells or organelles which are not readily labeled in vivo with appropriate radioisotopes (e.g., ^{3}H-thymidine), we have been developing methods for labeling of DNA, DNA bases, and their photoproducts after the completion of experimental manipulation of intact cells. The basic procedure is the introduction of radioactivity into the products of hydrolyzed DNA by methylation with tritiated dimethyl sulfate (DMS). For the method to be of general utility, three criteria must be met: (1) conditions must be such that the methylation reactions go to completion, so as to avoid problems arising from differential rates of labeling, (2) the reaction must be carried out with minimal manipulation, so that large numbers of samples can be processed, (3) the methylation products must be readily separated from each other as well as radioactive products (e.g., $NaCH_3^3SO_4$) which are of no interest.

We have succeeded in meeting these criteria for the identification of thymine and thymine-containing photodimers. The technique will be used to study the photobiology of "unlabelable" DNA such as mitochondrial DNA from *Neurospora*.

29.25 A COMPUTER PROGRAM FOR SOME BIOLOGICAL PROBLEMS

V. A. Singletary[1] J. E. Donnellan, Jr.

Many biological problems consist of a series of measurements which must be normalized to a common level, such as the percentage each measurement contributes to the total. Usually the experiment will consist of several sets of measurements which must be normalized and compared. Typical examples of these types of experiments are chromatographic separations where the variables are measured by the type(s) of radioactive label they contain or gradient centrifugation where again the measurement is by isotopic means. A computer program has been written whereby many sets of data may be analyzed consecutively and the necessary computations for separation of multiple variables performed. An option to plot the resulting data on linear or logarithmic scales is provided.

The program consists of an initial stage whereby certain values constant to the whole set of measurements are read into the computer. These constants might be the background reading for a series of counts in a scintillation counter and the isotopic spillover corrections in experiments using up to three radioactive labels. The next set of instructions involves the mathematical operation to be performed on each set of data and will differ for each set. This information tells the computer where the set of data begins and where it ends and whether a plot of the results is required. This phase of the program also chooses the isotope(s) of interest when multiple variables are being measured. The final stage of the program reads the experimental data into the computer, beginning and ending as determined by the information supplied at the second stage. The data are then computed, tabulated, and plotted as required, and upon completion a second set of data is read in, computed, tabulated, etc.

At the present time all information, both instructions and data, may be presented to the computer either in the form of punched cards or from magnetic tape. The magnetic tape is prepared from paper tape which has been automatically punched by the scintillation counter during data collection. The use of paper tape saves some 30 hr over the time required to have the data manually transcribed onto punched cards. Typically we receive the plotted data from the computer within 24 hr after the scintillation counting has ended.

Reference

[1] Mathematics Division.

30. Cooperative Programs

AEC-NCI COCARCINOGENESIS

Biochemistry of Carcinogenesis (30.1, 30.2)

E. S. Rogers[a]
Barbara R. Bussell
Brenda R. Cary
W. F. Fox
D. W. Parsons
T. J. Stephens, Jr.
Mary H. Welch

Enzymology of Chemical Carcinogenesis (30.3–30.8)

G. D. Novelli[a]
Audrey N. Best[a]
Lawrence Rosen[a]
L. C. Waters[a]
C. J. Wust[a]
Wen-Kuang Yang[a,b]
Ugo Del Monte[a,b]
Beryl Ortwerth[a,c]
Shigemi I. Simms[a]

Mammalian Chemical Carcinogenesis (30.9–30.12)

A. C. Upton[a]
N. K. Clapp[a,d]
J. W. Conklin[a]

Chromosomal Effects of Chemicals and Radiation (30.13, 30.14)

E. H. Y. Chu[a]
E. G. Bailiff[a]
Patricia A. Brimer[a]
Patricia L. Wortham[a]

Biophysics of Carcinogenesis (30.15–30.27)

R. B. Setlow[a]
J. R. Einstein[a]
F. C. Hartman[a]
J. W. Longworth[a]
C. H. Wei[a]
Maria D. C. Battista[a,e]
I. Lucille Norton[a]

Chemical Mutagenesis in Microorganisms (30.28–30.30)

F. J. de Serres[a]
H. V. Malling[a]
D. S. Carroll[a]
J. S. Wassom[a]

Viral Leukemogenesis (30.31, 30.32)

R. L. Tyndall[a]
Ernestine Teeter[a]

Somatic Cell Genetics (30.33, 30.34)

J. D. Regan[a]
W. H. Lee[a]

Inhalation Carcinogenesis (30.35–30.43)

D. G. Doherty[a]
J. O. Brick[a]
M. G. Hanna, Jr.
Paul Nettesheim
C. T. Bahner[d]
Alfred Hellman[f]
R. F. Newell[f]
W. L. Dake
C. D. Farmer
Mary W. Francis
J. L. Grey
Anna S. Hammons
Renfro Henderson
D. H. Martin
T. L. Penson
Leona C. Peters
R. W. Presley
J. H. Rather
R. C. Satterfield
B. E. Sise
A. K. Szakal
A. E. Thomas
J. R. Webb
J. E. Whittlesey
Mary L. Williams
Helen L. Zang
J. G. Farrelly[a,g]

Biochemical Regulation (30.44–30.48)

F. T. Kenney[a]
W. D. Wicks[a]
J. L. Wittliff[a,h]
C. B. Hager[a,i]
J. R. Reel[a,i]
G. R. Holloway[a]
Joseph Kendrick[a]
L. E. Roberson[a]

[a]Dual assignments
[b]Visiting investigator from abroad
[c]SRC/NATO Postdoctoral Fellow
[d]Consultant
[e]On leave of absence
[f]Loanee
[g]Biology Division Postdoctoral investigator
[h]USPHS Postdoctoral Fellow
[i]American Cancer Society Postdoctoral Fellow

30.1 TRANSMISSION OF SYNTHETIC GENETIC INFORMATION BY USE OF VIRUS VECTORS

Stanfield Rogers

Efforts to build and transmit synthetic genetic information using viruses are an outgrowth of our findings that the Shope papilloma virus carries the information for the synthesis of an arginase. Infection with this virus thereby causes a reduction in the arginine blood level of certain infected animals, including rabbits, mice, rats, and man. It seemed because of this that if the information for the synthesis of a specific enzyme could be added to the virus DNA or RNA, the information could be thereby transmitted. Our first efforts using the Shope virus DNA failed, as it was not possible to add information without breaking the continuous helix, which rendered the DNA noninfective. The recent report of Goulian, Kornberg, and Sinsheimer,[1] who used ligase to tie the ends of a continuous DNA together, suggests that this now should be possible. In any event, because of this difficulty, we went to an infective linear RNA derived from the tobacco mosaic virus. By use of polynucleotide phosphorylase it has been possible to add sequences of poly-A. Tobacco plants infected with this modified RNA reveal little loss of infectivity of the RNA. Following separation of the basic peptides from extracted tobacco leaves, and the subsequent separation of polylysines from the other peptides using a Beckman PA 35 resin, and their subsequent fractionation using carboxymethyl cellulose, it has been possible to isolate and purify trimeric, tetra-, and pentalysine from plants infected with the poly-A-modified TMV-RNA. No polylysines have been detectable in extracts of normal plants, normal TMV-RNA-infected plants, or plants inoculated with a mixture of poly-A and normal TMV-RNA.[2] This model indicates that more meaningful sequences such as that for the synthesis of an enzyme can be added to a virus nucleic acid and thereby use the virus as a vector to transmit the information. Such modified viruses have the potential of curing certain of the genetic deficiency diseases and may prove useful in the therapy of cancer and certain other diseases.

As infective DNA or RNA is unlikely to produce systemic infection in mammals, efforts are under way to add synthetic protein coats such as polylysine to the modified nucleic acid to protect it from extracellular nucleases in such animals. Another possibility that is being explored is the use of subunits of normal virus protein coat in a similar way. The protective effect of TMV protein coat subunits on the Shope papilloma virus DNA is currently being tested.

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30.2 REGULATION OF THE BLOOD LEVEL OF SPECIFIC METABOLITES THROUGH THE USE OF CHRONIC DIALYSIS AGAINST SPECIFIC ENZYMES

Stanfield Rogers

Repeated dialysis of the blood of patients with renal failure using artificial kidneys is well known. One of the problems of such machines is the metabolite imbalance caused by long-term dialysis. It occurred to us that such imbalance would be circumvented should the dialysis system be not allowed to act as a kidney but rather allowed to dialyze back into the blood stream. The normal kidneys of the animal or individual will then control the excretion following specific degradation of metabolites by enzymes on the exterior of the membrane. Current work in a number of laboratories is concerned with the control of metabolite blood levels through enzyme injection. Such methods are fraught with the danger of not only how much and how often to give the enzyme but because of the often fatal protein sensitivities developing upon repeated injection in time. These difficulties would be circumvented through the use of this modified form of chronic dialysis. This approach should aid in the specific therapy of cancer of various sorts as well as in the specific therapy of those with diseases such as histidinemia, this latter associated with the genetic lack of the specific enzyme histidase. A mini-Klug kidney has been kindly loaned by Dr. Converse Peirce of the Emory University School of Medicine. Our pilot studies are being done with the Shope rabbit papillomas, known to require large amounts of arginine. Arginase is the enzyme being dialyzed against.

In this system the rabbit blood arginine level may be drastically lowered with 4 to 6 hr dialysis against arginase. In rabbits repeatedly dialyzed over a period of days or dialyzed and maintained on a diet deficient in arginine, the Shope papillomas undergo a dramatic regression. As these tumors sometimes undergo a spontaneous regression, as a control the animals with greatly reduced tumor size were taken off the dialysis

and synthetic diet and full fed. The tumors then rapidly returned, indicating that the regression was caused by the therapy and not spontaneous.

30.3 USE OF VIRUSES AS CARRIERS OF ADDED GENETIC INFORMATION

Peter Pfuderer[1] Stanfield Rogers

Tobacco mosaic virus (TMV) RNA has been successfully modified by the attachment of polyadenylic acid (poly-A) moieties at the 3′ end, using ADP, polynucleotide phosphorylase, and the TMV RNA as primer in the reaction. This should lead to the production of polylysine in the infected host, either as a free polymer or attached to a viral protein.

Concomitantly, a chemical purification scheme has been developed which permits the isolation and determination of pure polylysine oligomers in the range from 1 to 30 from extracts of tobacco plants. Control runs on extracts of uninfected tobacco plants, normal TMV-RNA-infected tobacco plants, and poly-A or mixtures of poly-A and TMV-RNA-infected tobacco plants have failed to show any polylysine production in these extracts.

The poly-A-modified viral RNA, however, has been shown to produce appreciable amounts of polylysine. The polylysines produced ranged from 2 to 6 monomers in length, while the poly A added to the viral RNA was roughly 20 monomer units long. No change has been found in the protein coat of the new virus, and the polylysines seem to be produced unattached to any viral protein.

After the virus has been propagated by systemic infection for a second or third generation in the Turkish tobacco host as described above, it loses its ability to produce polylysine. Reasoning that this loss was due to a gradual takeover of wild-type unmodified virus present with the poly-A virus, an attempt was made to get a pure clone of the modified virus. The poly-A-modified RNA was purified on a sucrose density gradient, and fractions from the gradient were plaqued on a Xanthi tobacco host, which gives localized lesions typical of the mosaic infection. These plaques, which should be results of single viral RNA infections, were then reinfected and followed through several generations. To date, three of these clones have been found which produce a single polylysine polymer and seem to breed true through several generations.

Reference

[1] Enzymology section.

30.4 CHROMATOGRAPHIC COMPARISONS OF ISOACCEPTING tRNA's BETWEEN L-M CELLS IN IN VIVO CULTURE AND L-M-CELL-INDUCED TUMORS IN C_3H MICE

Wen-Kuang Yang D. H. Martin
Alfred Hellman Kiki B. Hellman[1]
G. David Novelli

As a continuation of the study of the tRNA's of L-M cells and L-M-cell-induced tumors,[2] a detailed chromatographic comparison of isoaccepting tRNA's has been carried out to determine the effect of drastic changes in the nutritional environment on the tRNA pattern in these cells. The tRNA's were isolated from washed L-M cells and from fresh solid tumor tissue. These were aminoacylated with homologous aminoacyl-tRNA synthetase preparations with the same amino acid, but carrying different labels (^3H and ^{14}C), and then co-chromatographed on the RPC-2 column.[3] The comparison has been made for 16 amino acids – serine, tyrosine, aspartate, phenylalanine, leucine, lysine, arginine, methionine, histidine, glycine, valine, alanine, isoleucine, tryptophan, proline, and threonine. Similar chromatographic patterns were observed for leucyl-, argininyl-, lysyl-, methionyl-, glycyl-, valyl-, tryptophanyl-, and prolyl-tRNA's. Significant quantitative differences in isoaccepting tRNA peaks were observed in species accepting isoleucine, alanine, threonine, and serine. Marked differences were found for tyrosyl-, phenylalanyl-, aspartyl-, and histidyl-tRNA's. A tyrosyl-tRNA migrating in front of the four tyrosyl-tRNA's which were resolved on the RPC-2 column was very prominent in L-M tumors but was completely absent in the L-M cells. Of the two phenylalanyl-tRNA's detected, the first peak was about 60% in L-M cells and below 3% in L-M tumors, whereas the second peak was about 40% in L-M cells and more than 95% in L-M tumors. The L-M cells showed two aspartyl-tRNA's, a major one and a minor one (80 and 20%); L-M tumors showed four aspartyl-tRNA's, two (8 and 2%) migrating at the same chromatographic positions as those of L-M cells and two distinct peaks (75 and 15%) migrating at different positions where no aspartyl-tRNA's from L-M cells were observed. The L-M tumors had three histidyl-tRNA peaks, of which the most prominent one in front was detected as the most minor one in the L-M cells. The alterations of chromatographic patterns of these aminoacyl-tRNA's between the L-M cells and the L-M tumors were demonstrated either by using synthetase preparations from the same source of cells and tissues or by reversing the enzyme preparations for the pre-column aminoacylation.

Further experiments showed the following facts:

1. Although tumors from the male and the female C_3H mice were found to have some differences in the growth and regression rate, no differences were detected in their tyrosyl- and aspartyl-tRNA's.

2. When the tumor tissue was trypsinized for dispersion, adapted again to in vitro culture by one passage in 19% horse serum–medium 199 and several passages in 0.5% Bacto-peptone–medium 100 as a monoloayer, and finally grown in suspension culture to obtain enough material for isolating tRNA, the chromatographic patterns of tyrosyl-, aspartyl-, and phenylalanyl-tRNA's of these in vitro grown L-M tumor cells showed those of L-M cells instead of those of L-M tumors.

3. When 5% mouse serum or 5% horse serum was included in the culture medium in the place of 0.5% Bacto-peptone for three passages, no conversion of aspartyl- and tyrosyl-tRNA's into the tumor pattern was observed.

4. Co-chromatography of tyrosyl-, aspartyl-, seryl-, and phenylalanyl-tRNA's from C_3H mouse liver with those from L-M cells or L-M tumors revealed that the isoaccepting aminoacyl-tRNA patterns of L-M tumors and the liver were similar.

5. Since tRNA's were prepared from L-M cells which were in cell suspension and from L-M tumors which were solid tissue, the possibility arose that different physical treatment of the source of material before phenol extraction might alter the chromatographic properties of isoaccepting tRNA's. This possibility was excluded by the finding that isoleucyl-, valyl- and seryl-tRNA's prepared from the cell suspensions of C_3H mouse reticulocytes were similar to those of L-M tumors in chromatographic separation.

To summarize, the results showed that the multiple isoaccepting tRNA's undergo remarkable changes in L-M cells following the drastic change from the in vitro cell culture to the in vivo environment. Whether this phenomenon results from the selection of the cell clone population or is due to adaptation of the cells at the cytoplasmic level remains to be determined.

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30.5 ETHYLATION OF HEPATIC tRNA BY ETHIONINE

Lawrence Rosen Shigemi I. Simms
G. David Novelli

Ethylation by ethionine, a specific hepatocarcinogen, of liver RNA differs in two obvious ways from other unnatural agents which alkylate liver tRNA. It alkylates only tRNA to any significant extent and yields a greater variety of alkylated products. The amount of ethylation which occurs is distributed equally between the purine and pyrimidine fractions.

Ethionine is the ethyl analog of methionine, the endogenous source of methyl groups in tRNA. For this reason methylation studies with methionine were also done. Different ethylpurines and pyrimidines were synthesized and characterized to make positive identifications. The results reported below demonstrate some qualitative similarities between ethionine and methionine but also show marked differences. This demonstrates that ethionine has a different specificity and does not randomly compete with methionine for the same sites in tRNA.

In the purine fraction, only guanine is ethylated, not adenine. There are four different ethylguanines, three of which have been identified.[1] N^2-Ethyl, N^2,N^2-diethyl-, and 7-ethylguanine account for 23, 2, and 7% of the total ethylation respectively; two-thirds of the purine ethylation. An unidentified ethylpurine, probably another ethylguanine, based on electrophoretic studies, accounts for 17% of the total ethylation. These results contrast with those obtained with methionine; in the latter case both guanine and adenine are methylated.

The major ethylated purine, N^2-ethylguanine, apparently has a shorter half-life than the other ethylated purines in tRNA. This also contrasts with methylated purines, of which 7-methylguanine apparently has the shortest half-life.[2]

Approximately 80% or more of the ethylation which occurs in the pyrimidine nucleotide fraction is accounted for by 2′-*O*-ethylation of the ribose moiety. Paper chromatography demonstrates there are not less than three different 2′-*O*-ethylpyrimidine nucleotides in rat liver tRNA, and they appear to be uridylic acid derivatives, as indicated by electrophoresis. One may be assumed to be 2′-*O*-ethyluridylic acid, and the others could conceivably be 2-*O*-ethyl derivatives of minor bases related to uridylic acid such as ribothymidylic acid, pseudouridylic acid, etc. These compounds differ markedly from those obtained with the methylated pyrimidine nucleotide fraction. In the latter case,

2′-*O*-methylation accounts for 12% (maximum) of the total methylation found. The major methylated pyrimidine nucleotides are ribothymidylic and 5-methylcytidylic acids, whereas only a small amount of 5-ethylcytidylic acid has been identified.

The above results indicate that in tRNA, only guanine in the purine fraction and mainly uridylic acid (and/or its derivatives) in the pyrimidine fraction are ethylated to a major extent. For the former, only base ethylation occurs, and for the latter, mainly 2′-*O*-ethylation occurs. These findings indicate a high specificity for ethionine, different from methionine.

References

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30.6 *E. COLI* tRNA: BEFORE AND AFTER BACTERIOPHAGE INFECTION

L. C. Waters G. David Novelli

When *E. coli* is infected with T2 or T4 bacteriophage, its leucine tRNA is altered. As judged by reversed-phase column chromatography the leucyl tRNA of the cells is altered quantitatively early after infection (early change[1]). Moreover, a further qualitative alteration is observed late in infection and is characterized by the appearance of one or more "new" peaks of leucine tRNA (late change[2]).

Concerning the early change, we have repeated and expanded the experiments mentioned in the previous progress report and have concluded that it results from an apparently specific inactivation of only one of at least five isoaccepting leucine tRNA's found in normal cells.[3] This inactivation does not appear to be due to a selective loss of –CCA termini nor a denaturation of the type described by Lindahl *et al.*[4] We have proposed that this early change is the result of a specific enzyme, probably a nuclease, which is phage induced. However, to date we have failed to demonstrate such an activity in cell-free extracts from phage-infected cells.

Our efforts to elucidate the origin of the "new" leucine tRNA's are continuing. In vitro labeling experiments, particularly with ^{35}S, strongly suggest that the "new" leucine tRNA's are products of *de novo* synthesis. However, these same experiments suggest that other tRNA's must also be synthesized after phage infection; that is, the specific activity of tRNA labeled after phage infection is 25% of that of a sample from normal cells labeled for the same period of time. A recent report[5] presents evidence that a considerable quantity of the nucleic acid synthesized after phage infection is host specific, although host protein synthesis is shut off immediately. In light of this report, labeling experiments as just described must be interpreted with caution. The effects of chloramphenicol (CAP) and sodium azide on the formation of the "new" tRNA's have been investigated. Chloramphenicol does not inhibit the formation of the new peaks. Sodium azide, though expected to inhibit macromolecular synthesis, did not inhibit the formation of the new peaks; however, two intermediate peaks were formed. Sodium azide did not affect the profile of tRNA from normal cells. The significance of this observation is not clear.

The control experiments using CAP have yielded some interesting results. First, in the presence of CAP, tRNA synthesis continues almost linearly with time up to at least 4 hr. This tRNA appears to have full leucine acceptor activity, a result which is in agreement with a report of Ezekiel and Valulis.[6] Second, and most interesting, is the observation that the chromatographic profile is markedly altered, similar in appearance to undermethylated tRNA's. In vitro methylation shows that the CAP tRNA is not undermethylated when compared with the usual undermethylated tRNA as obtained from a relaxed strain of *E. coli* after methionine deprivation. We are currently investigating the possibility that this CAP tRNA is defective in only one or a few of the modifications known to occur at the polynucleotide level, for example, methylation or thiolation. If so, such tRNA might prove valuable in elucidating the functional significance of such modifications.

The biological significance of the "early" and "late" change in leucine tRNA following phage infection is unknown. Studies concerning this problem are in progress.

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30.7 MICROANALYSIS OF ISOACCEPTING tRNA's BY REVERSED-PHASE COLUMN CHROMATOGRAPHY

Wen-Kuang Yang L. C. Waters

An important role for tRNA in the process of cellular regulation has been implicated by the observation of multiple isoaccepting tRNA's in the organism and by the findings that they may be altered according to the state of cellular function. This has produced a need for a generally useful method for the analysis of isoaccepting tRNA's. In this laboratory, a standard method has been devised for this purpose by employing the reversed-phase columns which were originally developed in the macromolecular separations program for the large-scale isolation of pure tRNA species. With this method and the use of precharged tRNA, sample sizes in the range of one A_{260} unit can be analyzed for the isoaccepting tRNA species of an amino acid. The main features of the method are summarized as follows:

1. tRNA is prepared from cell suspensions, fresh tissues, or frozen samples by phenol and 1 *M* NaCl extraction followed by DEAE-cellulose column chromatography.
2. Active aminoacyl-tRNA synthetases are prepared from fresh or frozen samples by subcellular fractionation, DEAE-cellulose column chromatography, and Sephadex G-100 gel filtration.
3. Conditions and criteria for satisfactory aminoacylation of the tRNA with ^{14}C- or ^{3}H-labeled amino acids are determined using the paper disk assay.
4. The labeled aminoacyl-tRNA is prepared for subsequent reversed-phase column chromatography by direct isolation from the reaction mixture on miniature DEAE-cellulose columns.
5. The samples to be analyzed are differently labeled (^{3}H or ^{14}C), mixed, and subjected to reversed-phase column chromatography. Chromatographic conditions, such as flow rate, temperature, additives (such as divalent ion, reducing agents, buffer, etc.), and NaCl gradient, are optimized to give the best recovery and resolution.
6. Columns are assayed by TCA precipitation of the individual fractions (in the presence of carrier DNA), collection on Millipore filters, and subsequent radioactivity measurements.
7. A qualitative comparison of the isoaccepting tRNA's can be made directly from the counting data. With the information obtained in feature 3, a direct quantitative comparison can be made.

This method has been applied to bacteria, fruit fly, liver and gill tissues of fish, toad liver, mouse plasma cell tumors, tissue culture L-M cells and L-M tumors, and the mouse reticulocytes. It was especially useful in the cases where the source of material for preparing tRNA was limited.

30.8 STUDIES ON THE PURIFICATION AND CHARACTERIZATION OF tRNA PYROPHOSPHORYLASE

Audrey N. Best G. David Novelli

The purification of tRNA pyrophosphorylase has been repeated several times and extended in a large-scale process. The procedure, which has been outlined previously,[1] was modified to include a batch hydroxylapatite step after the $MnCl_2$ heat treatment. This step eliminates about 90% of inactive A_{280} material and makes possible the chromatography of 12,000 A_{280} units on the first 5 × 80 cm DEAE-cellulose column. Repeated chromatography on another DEAE-cellulose column and on a final hydroxylapatite column gives about 10 mg of purified enzyme from 3.25 kg of *Escherichia coli* B cells. The largest single loss of enzyme units occurs during or after the batch hydroxylapatite treatment, but this step appears to be necessary for large-scale work. The product of this purification compares well with smaller-scale preparations. Since no other purification steps we tested were promising, we decided to use this preparation for our enzymatic studies.

We were able to see five fairly sharp protein-staining bands by electrophoresis of the final fraction on cellulose acetate membranes in 0.02 *M* phosphate buffer, pH 6.35. Three of these bands (cut out from corresponding nonstained samples) showed incorporation of both AMP and CMP into a partially degraded tRNA preparation. It is not clear just how pure our preparation is, but we believe that this indicates one enzyme can incorporate both nucleotides. There has been no evidence of subunits during our purification procedure, as the activity always occurs in one peak.

Our final preparation did not inhibit the aminoacylation of a purified tRNA by its specific synthetase, and preliminary tests did not show aminoacyl tRNA synthetase activity. The preparation is free of acid and alkaline phosphatase activity with *p*-nitrophenyl phosphate as the substrate, but at very high concentrations it does show $CoCl_2$- and $CaCl_2$-stimulated activity at pH 6.5 toward bis(*p*-nitrophenyl) phosphate and ATP. This might indicate a contamination with the 5′-nucleotidase described by Neu,[2] and osmotic shock

treatment of the bacterial cells in the enzyme extraction step could eliminate this contamination. However, under our assay conditions for AMP (CMP) incorporation these activities are not a problem.

Our assay procedure consisted in incorporation of radioactive AMP (from ATP) into a partially purified mixed tRNA preparation from *E. coli* W (prepared by periodate oxidation of the terminal adenosine followed by amine cleavage and alkaline phosphatase treatment). This altered tRNA preparation can be repaired to about 1200 $\mu\mu$moles of AMP per A_{260} tRNApCpC. Maximum activity is noted at pH 9.5 in Bicine buffer [*N,N*-bis(2-hydroxyethyl) glycine] with 2×10^{-3} *M* ATP and about 35×10^{-6} *M* tRNApCpC (1 $A_{260} = 10^{-6}$ *M* tRNA). Under initial velocity conditions at 30°C (2-, 4-, and 6-min assay) the K_m for ATP is 1.6×10^{-4} *M*, and the K_i for CTP is 1.8×10^{-4} *M*. The K_m for the mixed tRNApCpC is 7×10^{-6} *M*. We plan to extend these studies by removing the second nucleoside from this preparation and measuring the K_m for CMP incorporation with the corresponding K_i for ATP. These experiments require rather large amounts of tRNA. It would be interesting to see if the K_m for tRNApCpC(or pC) varies with different tRNA preparations. Initial velocity rates can be increased (using optimum ATP and tRNApCpC concentrations) with increasing levels of β-mercaptoethanol up to 0.1 *M*. However, equilibration of the enzyme for at least 6 min at 0.02–0.1 *M* β-mercaptoethanol at the incubation temperature of 30° results in initial velocity values which are more linear over a fivefold increase in enzyme concentration.

We are examining several highly enriched and specific tRNA preparations which have been purified by the Chemical Technology Division, Macromolecular Separations Program, to see if the terminal nucleosides can be removed chemically without damage to the functional tRNA structure. We would like to see full charging activity by the specific synthetase after the treated tRNA has been repaired by the tRNA pyrophosphorylase. We know that $tRNA^{Phe}_{E.\ coli}$ is damaged to a certain extent by the periodate treatment. We are interested in determining under carefully controlled conditions which nucleotides can be incorporated into purified tRNA after sequential removal of the four or five terminal nucleosides from the 3′-OH terminus.

We are planning to examine the effect of enzyme and chemical treatment of purified tRNApCpC substrate in order to gain some insight into the recognition site(s) of the tRNA pyrophosphorylase for this substrate. The reverse action of the enzyme, pyrophosphorolysis of tRNA, will be studied in regard to its effectiveness with precharged tRNA.

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30.9 FURTHER OBSERVATIONS ON THE COMBINED CARCINOGENIC EFFECTS OF X RAYS AND TRIETHYLENEMELAMINE IN MICE

A. C. Upton W. D. Gude[1] J. W. Conklin

Introduction. – The radiomimetic alkylating agent triethylenemelamine (TEM) resembles x rays in certain late somatic effects on RF mice. These include shortening of the life-span, leukemogenic effects on the thymus, induction of tumors in the lung, ovary, and other organs, and the formation of lens opacities. The present study was undertaken to explore the combined effects of TEM and x rays administered concurrently, with the aim of determining whether the effects of the two agents would be additive to a degree consistent with a common mode of action.

Results and Discussion. – Ten-week-old female RF/Un mice were subjected to: (1) intraperitoneal injection of TEM, 0.25–4.0 mg/kg; (2) 25–500 r whole-body 300-kvp x irradiation (80 r/min); or (3) both TEM and x irradiation (TEM injected 1 to 5 min before irradiation). Preliminary results 30 months after treatment show that each agent given alone shortened the life-span but that the effect was greater in mice receiving both agents than in those receiving a comparable total dose of either agent alone, especially at high dose levels. Likewise, each agent alone induced thymic lymphomas and ovarian tumors, but when both agents were administered together, the incidence exceeded that induced by either agent alone, indicating that the two agents administered together exerted effects which were additive, if not synergistic, depending on the end effect in question. Since, however, a few of the mice remain alive and the lesions induced by the two agents remain to be analyzed in full, the significance of their combined effects is yet to be determined.

To explore possible mechanisms of synergistic effects of the two agents, the combined toxicity of the agents to thymus and hemopoietic cells is being analyzed with respect to effects on thymus weight and on killing of hemopoietic cells, as judged by the Till-McCulloch spleen nodule method. Preliminary results imply that x rays and TEM differ quantitatively, if not qualitatively, in their effects on blood-forming cells.

Reference

[1] Pathology and Physiology section.

30.10 ONCOGENICITY OF METHYL METHANESULFONATE (MMS) IN MALE RF MICE

N. K. Clapp R. E. Toya, Sr.[1]
A. W. Craig[2]

Introduction. – Alkylation of nucleic acids has been postulated to explain the mutagenic and carcinogenic action of certain alkylating agents; for example, dimethylnitrosamine (DMN) and diethylnitrosamine (DEN) alkylate nucleic acids and are potent carcinogens. Conversely, methyl methanesulfonate (MMS), which methylates DNA and RNA, has not been reported to be carcinogenic hitherto.

Results and Discussion. – In a total of sixty-three 11-week-old male mice from a noninbred subline of the RF/Un strain, which received 30 mg of MMS per kilogram body weight daily in the drinking water throughout life, we found an increased incidence of lung tumors (70.2% compared with 38.9% in 162 controls) and thymic lymphomas (14.9% compared with 3.7% in controls). As measured by lung tumor induction, therefore, MMS appears to resemble DEN and DMN. DEN and DMN fail to induce leukemia, however, whereas MMS appears to resemble x rays, nitrogen mustard, TEM, and Myleran – all of which induce thymic lymphomas in RF mice.

Since alkylation occurs primarily at the 7-position of guanine in both DNA and RNA after MMS, DMN, and DEN, differences in oncogenicity between MMS, DMN, and DEN suggest that a qualitative difference may exist between the site(s) of alkylation by these agents within nucleic acids, the precise location of the alkylation along the nucleic acid chain possibly being important in the oncogenic process. Studies to determine the cocarcinogenicity of MMS with DMN and DEN are now in progress.

References

[1] Mammalian Recovery section.

[2] Paterson Laboratories, Christie Hospital, Manchester, England.

30.11 RELATIONSHIPS BETWEEN CUMULATIVE DOSE OF DIETHYLNITROSAMINE (DEN) AND INCIDENCE OF TUMORS IN RF MICE

N. K. Clapp R. E. Toya, Sr.[1]
A. W. Craig[2]

Introduction. – Diethylnitrosamine (DEN) is a potent carcinogen in a number of species of animals, inducing a high incidence of liver hepatomas, lung adenomas, and squamous cell carcinomas of the forestomach in RF mice. To determine tumor-incidence–cumulative-dose relationships, 450 eight- to ten-week-old male RF mice were divided into six different groups and given DEN in the drinking water in daily doses of 2, 3.5, 6, or 11.5 mg per kilogram body weight for treatment periods of varied lengths; cumulative doses ranged from 57 to 943 mg per kilogram body weight.

Results and Discussion. – Mean survival times for the various dose groups were markedly reduced in groups receiving high total doses and in groups where treatment periods (and drug toxicity) extended for several months. The incidence of liver tumors increased linearly with increasing cumulative dose throughout the range of doses used. In contrast, maximum incidences of lung and stomach tumors (~80 and 100% respectively) were reached at low cumulative doses, and low thresholds were suggested, while none was seen for liver oncogenesis. In three groups that received DEN at the same daily dose (6 mg/kg) but with varied treatment periods (1, 7, and 22 weeks), the higher-dose groups developed the highest incidences of tumors, despite decreased mean survival time. At the lowest dose, lung tissue would appear most sensitive, stomach intermediate in sensitivity, and liver least sensitive to DEN carcinogenesis.

References

[1] Mammalian Recovery section.

[2] Paterson Laboratories, Christie Hospital, Manchester, England.

30.12 PRELIMINARY INFORMATION ON THE OCCURRENCE OF ETHER-LINKED LIPIDS IN TUMORS INDUCED BY DIETHYLNITROSAMINE

N. K. Clapp F. Snyder[1]

Introduction. – Previous studies have indicated that most neoplasms of animal and human origin contain increased levels of alkyl and alk-1-enyl glyceryl ether diesters and ether-linked phosphoglycerides.[2] The purpose of this study was to determine the occurrence of glyceryl ethers in chemically induced tumors of various stages of malignancy.

Results and Discussion. – Six male RF mice which were given diethylnitrosamine (DEN) in the drinking water (6 mg per kilogram body weight per day for seven weeks) developed lung adenomas, liver hepatomas, and squamous cell carcinomas of the forestomach; these were examined histologically and lipid analyses were performed. Normal nontreated tissue and nontumorous tissues from mice given DEN were analyzed similarly.

The tumors induced by DEN contained substantial quantities of ether-linked lipids, in contrast to very low or undetectable amounts in control and treated non-tumorous tissue. The ether-linked lipids in the neutral glyceride fraction of the stomach tumors appear as glyceryl ether diesters on thin-layer chromatograms. Stomach tumors which appeared more malignant histologically also contained higher amounts of glyceryl ether diesters. Lung and liver tumors were not large enough to yield adequate lipids for complete analysis. Further studies are in progress to confirm the impressions obtained in these preliminary results.

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[2] F. Snyder and R. Wood, *Cancer Res.* **28**, 972–78 (1968).

30.13 CHEMICAL INDUCTION OF FORWARD AND REVERSE MUTATIONS IN CHINESE HAMSTER CELLS IN CULTURE

E. H. Y. Chu Patricia A. Brimer H. V. Malling

Introduction. – In previous reports,[1,2] we summarized results demonstrating the selection of biochemical mutants from Chinese hamster cells in tissue culture and the induction of forward and reverse mutations at these loci with ethyl methanesulfonate (EMS). These studies have been extended to include other chemical mutagens as well as to define the conditions of mutation expression.[3] The present experiments were designed to elucidate, if possible, the molecular mechanism of gene mutation in the hamster cells, based on reverse mutation tests with compounds whose specific mutagenic action is well established in microorganisms.[4-6]

Methods. – The chemical mutagens employed in this study were EMS, MMS (methyl methanesulfonate), MNNG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine), and ICR-170 (2-methoxy-6-chloro-9-[3-(ethyl-2-chloroethyl)aminopropylamino]acridine dihydrochloride). Either spontaneously occurring or chemically induced mutant cell clones that were resistant to 8-azaguanine (azg^r) were treated with the above mutagens to induce reversions to drug sensitivity (azg^s). Prior to the reversion experiment, the possible background in the cell populations of spontaneous azg^s revertants was removed by growing cells in the presence of 30 μg/ml azaguanine. After mutagen treatment, the cells were washed and incubated for 42 hr before the selective agents were added to the medium. In this selective medium, which contained aminopterin, hypoxanthine, thymidine, and glycine, azg^s cells can grow whereas azg^r cells cannot. Both cell survival and mutation frequency were determined in terms of colony formation.

Results and Discussion. – A highly significant increase over the controls in the forward mutation frequency from azg^s to azg^r can be induced with all four mutagens. In equimolar concentrations and under similar experimental conditions, the relative effectiveness of the four compounds used is as follows: ICR-170 > MNNG > MMS > EMS.

A number of mutant clones were isolated which were shown to fully retain the resistant character after numerous cell generations in the absence of the drug. Reversion to drug sensitivity occurred either spontaneously or following mutagenic treatment (Table 30.13.1). The limited number of mutant clones tested did not permit definite conclusions to be drawn concerning the mutagenic specificity of various compounds in hamster cells. Nevertheless, several interesting facts are already evident:

1. There appear to be qualitative differences in revertibility to azg^s among the class of azg^r mutants which originated spontaneously, those induced by EMS or MMS, and those induced by ICR-170.
2. Some of the EMS- or MMS-induced mutants reverted either spontaneously or very significantly after MNNG treatment. In cases where both EMS and MNNG seemed to induce reversions, MNNG invariably was more effective than EMS.
3. Of all the 12 mutants tested which were induced by ICR-170, none was able to revert significantly to drug sensitivity.

Resistance to azaguanine in hamster cells has been shown to be due to the loss of inosinic acid–guanylic acid pyrophosphorylase activity.[1] Reversion to drug sensitivity, that is, regaining of the enzyme activity, could be due either to back mutation at the locus or to suppressor gene(s). The present data on reversion induction with specific chemical mutagens reveal the complexity and qualitative differences between azg^r mutants of different origin. The genetic alterations in the revertible mutants are likely to be single base-pair changes. Furthermore, it seems possible that the genetic alterations in azg^r mutants which can revert after treatment with EMS and MNNG are base-pair substitutions. The nonrevertible mutants can be interpreted as multiple intragenic changes or deletions.

Table 30.13.1. Distribution of Azaguanine-Resistant Mutants in Chinese Hamster Cells According to Their Spontaneous or Induced Revertibility to Drug Sensitivity

Number of Mutant Clones	Genotype	Presumptive Origin	Revertibility ($azg^r \rightarrow azg^s$)			
			Spontaneous	EMS	MNNG	ICR-170
3	azg^r	Spontaneous	+	+	+	
3			–	+	+	NT
2			+	+	–	
2			–	–	–	
2	azg^r	EMS-induced	+	+	+	
2			–	+	+	
4			–	–	+	NT
0			–	+	–	
13			–	–	–	
4	azg^r	MMS-induced	+	+	+	
3			–	+	+	NT
2			–	–	+	
0			–	+	–	
9			–	–	–	
12	azg^r	ICR-170-induced	–	–	–	–

NT = not tested.

It is clear from these data that measurement of reverse mutation frequencies cannot be used as a screening method for testing of mutagenicity. These tests have to be performed with systems where quantitative measurements of forward mutation frequencies can be obtained, such as the induction of mutations from azg^s to azg^r.

References

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30.14 MUTAGENICITY OF CHEMICAL CARCINOGENS IN MAMMALIAN CELLS

E. H. Y. Chu E. G. Bailiff H. V. Malling

With the availability of genetic markers and the demonstration of chemical mutagenesis in tissue culture mammalian cells, it becomes feasible to test in the same system both carcinogenicity and mutagenicity of a particular agent. We have studied the cytotoxicity and photodynamic effect[1] of several carcinogenic and non-carcinogenic polycyclic hydrocarbons. This report summarizes the preliminary results on mutation induction in Chinese hamster cells with these same series of compounds. The mutagenic change studied was from sensitivity to 8-azaguanine (wild type) to resistance.

The compounds were first dissolved in dimethylformamide and added, at appropriate concentrations, to the cell cultures. The solvent at a final concentration of 1% in the medium reduced cell survival to about 70%, but was not itself mutagenic. Cells were exposed to various polycyclic hydrocarbons for 24 hr in the dark, at the level of concentrations that would permit normal or slightly reduced survival. Our results to date clearly show that 9,10-dimethyl-1,2-benzanthracene was highly mutagenic, whereas 1,2,3,4-dibenzanthracene and 1,2,5,6-dibenzanthracene caused slight but significant increase in the forward mutation frequency. On the other hand, neither 1,2-benzpyrene nor 3,4-benzpyrene was shown to be mutagenic. These results are similar to a parallel study in *Neurospora*, with the exception that in hamster cells both 1,2,3,4- and 1,2,5,6-dibenzanthracenes exhibit mutagenic activity.

The work has been extended to include other carcinogens, such as aromatic amines. Our aim is to test the possibility that metabolic derivatives rather than the test compound per se were responsible for the final

effect. It is hoped that studies along these lines may throw some light on the somatic mutation theory of carcinogenesis.

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30.15 PROTEIN CHEMISTRY

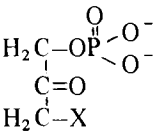
F. C. Hartman

Design and Use of Active-Site Specific Enzyme Reagents. – Chemical reagents can be endowed with a high degree of specificity for the active site of a given enzyme by incorporating into their structures those functional groups of the natural substrate required for binding to the enzyme in question. In addition to the usefulness of such reagents in basic studies concerning the structure and mechanism of action of enzymes, they are of potential value in cancer chemotherapy.[1]

In our last progress report,[2] the preparation of 1-hydroxy-3-iodo-2-propanone phosphate (HIPP) (I), a reactive derivative of dihydroxyacetone phosphate (DHAP) (II), was described. I assumed that HIPP would have an affinity for and be capable of reacting covalently with the active sites of triose phosphate isomerase (TPI), aldolase, and glycerophosphate dehydrogenase, enzymes for which DHAP is a natural substrate. During the past year, the corresponding bromo (HBPP) (III) and chloro (HCPP) (IV) compounds have been prepared, and a comprehensive investigation of their reaction with the aforementioned enzymes has been initiated.

Substantial evidence for the active-site specific modification of TPI by HIPP has been obtained and published,[3] and preliminary data indicate that HBPP reacts with TPI in a similar manner. These reagents will presumably permit structural analysis of at least a portion of the active site of TPI.

The inactivation of aldolase by HIPP is complex. Significant (50 to 70%) enzymic activity is recovered by treating the modified aldolase with cysteine or other sulfhydryl compounds. This observation, in addition to quantitative sulfhydryl determinations on the native and modified enzyme and the fact that HIPP oxidizes cysteine to cystine, suggests that the inactivation is, in part, due to the oxidation of sulfhydryl groups to disulfides. Approximately two moles of reagent per mole of aldolase are irreversibly incorporated and may account for the lack of complete restoration of activity induced by sulfhydryl compounds. HBPP and HCPP do not inactivate aldolase under the conditions used for the inactivation by HIPP.



X = I (I), OH (II), Br (III), Cl (IV)

A Heavy Atom Derivative of Rubredoxin. – The reaction of *C. past.* rubredoxin with ^{14}C-labeled α-(*p*-iodophenyl)-acetimidate has been investigated as a means of obtaining a derivative suitable for x-ray crystallographic studies.[2] Contrary to preliminary data, substitution occurs at various ϵ-amino groups, resulting in a mixture of derivatives which has resisted resolution by usual procedures. These difficulties and the fact that the elucidation by x-ray diffraction of the three-dimensional structure of rubredoxin is approaching completion in another laboratory[4] prompted me to terminate this study.

References

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[4] L. H. Jensen, University of Washington at Seattle.

30.16 MOLECULAR STRUCTURE OF THYMINE (*cis-syn*) PHOTODIMER

C. H. Wei J. R. Einstein

Ultraviolet irradiation of living cells damages the cellular DNA by causing bonds to form between neighboring pyrimidine residues. The thymine photodimer isolated from irradiated DNA has previously been shown to be a *cis-syn*-cyclobutane derivative. This photodimer may also be obtained by uv irradiation of frozen aqueous solutions of thymine, and was crystallized as the dipotassium and monosodium salts from alkaline alcohol-water solutions. Both forms are highly radiation sensitive, and both exhibited a slow, small change in unit-cell dimensions during data collection. Cell dimensions measured at about the middle of the data collection were, for the potassium salt, $a = 7.01$, $b = 25.97$, $c = 8.03$ A, $\beta = 99°10'$; for the sodium salt, $a = 6.56$, $b = 29.01$, $c = 6.76$ A, $\beta = 106°2'$. The space groups are *Cc* for the potassium salt, $P2_1/n$ for the sodium salt, with $Z = 4$ in both cases. The structure of the sodium salt ($C_{10}H_{11}O_4N_4Na \cdot H_2O$) was determined by the symbolic addition procedure and was based on 1817 independent reflections. It was shown

that x rays split the cyclobutane ring of the dimer and that the two resulting monomers fit well into the space previously occupied by the parent dimer, although individual atoms move as much as $1\frac{1}{2}$ A. The structure was assumed to consist of a random distribution of dimers and monomer pairs, having related site occupancies which change during data collection. This model has been refined, with the use of a rigid-body treatment for the monomers, to a present value of 0.053 for the reliability index $R_1(F)$, based on 1014 of the strongest reflections. The atomic positions determined prior to consideration of the low-occupancy monomer sites (roughly 25% occupancy for this set of data) represented averages of the dimer and monomer positions and gave incorrect bond distances, particularly across the cyclobutane ring. However, normal bond distances were obtained for the dimer from refinement of the mixed dimer-monomer model.

The potassium salt crystals are much more mosaic than the sodium salt, and only 523 reflections were used. Although no such detailed analysis was carried out as for the sodium salt, it is evident that the dimer has roughly the same configuration in the two crystals. The following discussion refers to the sodium salt of the dimer, a view of which is shown in Fig. 30.16.1.

The cyclobutane ring is buckled, with a dihedral angle of 28°. Both pyrimidine residues are in approximately an envelope configuration, with one atom of each ring several tenths of an Angstrom unit out of the least-squares best plane of the other five atoms. The best planes, calculated for the approximately coplanar atoms of each ring, make an angle of 59° with each other. When viewed along the normal to the plane bisecting the two best planes, the two pyrimidine residues are rotated from each other by an angle of about 40°. The molecule is unusually crowded, with a distance of only 3.0 A between the two methyl carbons (normal van der Waals distance, 3.5 to 4.0 A).

The crystal has a center of symmetry, and therefore there are two enantiomeric forms of the dimer, with right- and left-handed screw senses.

The crystal contains one water of hydration per dimer. A difference Fourier revealed all 11 hydrogen atoms. The proton replaced by the sodium ion has been removed from the N_3 atom of one pyrimidine residue; this nitrogen is coordinated to the sodium ion. The sodium is coordinated in a distorted octahedral configuration to atoms of four different dimers and to one water oxygen. Atoms of one dimer are involved in seven hydrogen bonds, four to four other dimers and three to three water oxygens. Each water is hydrogen bonded to three dimers and is coordinated to a sodium ion.

The pair of thymine monomers, which replace a dimer split by x rays, lie on nearly parallel planes spaced 3.4 A apart. Replacement of a dimer by a monomer pair is accompanied by the breaking of only two hydrogen bonds and the formation of one new hydrogen bond.

There is no free rotation about any bond in the photodimer; therefore the structure must closely approximate that of a corresponding moiety within a molecule of uv-irradiated DNA. One may attempt to explain the denaturation of DNA after uv irradiation by comparison of the dimer configuration with that of two neighboring thymine residues in the DNA helix. Several striking differences are observed. (1) In the DNA helix the distance between atoms N_1 and N_1' of neighboring thymines – the atoms bridged by sugar-phosphate-sugar – is 4.4 A, while in the dimer this distance is 2.9 A. Thus in DNA the configuration of the sugar-phosphate-sugar moiety would have to change radically on formation of the cyclobutane ring. (2) Neighboring thymine residues in DNA lie on nearly parallel planes spaced 3.4 A apart. The corresponding planes of the dimer are at an angle of 59° to each other. (3) Suppose the right-handed form of the dimer is introduced into the (right-handed) DNA helix in such a manner that the two pyrimidine planes are each tilted by about 30° to the equatorial plane of the helix, and so that the atoms

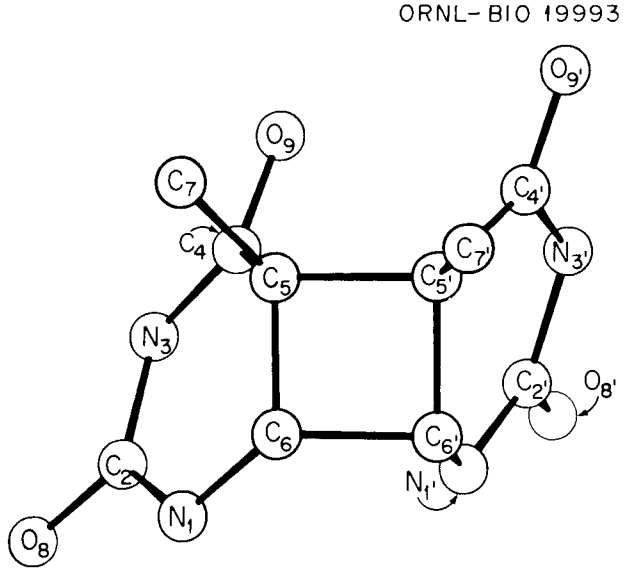

Fig. 30.16.1. The Sodium Salt of Thymine Dimer Viewed from a Direction Perpendicular to the Average Plane of the Cyclobutane Ring.

of one thymine residue lie in axial projection nearly over the corresponding atoms of one thymine residue of DNA. Then in the other pyrimidine the atoms which correspond to those forming interchain hydrogen bonds in the helix would be displaced by several Angstrom units from the required positions. Introduction of the left-handed dimer would change the screw sense of the helix. In summary, it is not difficult to rationalize the denaturation of DNA on formation of these cyclobutane dimers.

30.17 X-RAY STUDIES ON DIPEPTIDES: *N*-BROMO(AND CHLORO)ACETYL-L-PHENYLALANYL-L-PHENYLALANINE ETHYL ESTERS

J. R. Einstein C. H. Wei

In an attempt to obtain single crystals suitable for x-ray structural analysis, both bromo and chloro derivatives[1] were recrystallized from ethanol. Crystals of the bromo derivative are monoclinic with cell parameters a = 12.951 A, b = 17.617 A, c = 4.951 A, and β = 103.23°. The volume of the unit cell is 1100 A^3. The observed density of 1.39 g/cm^3 (by flotation) compares satisfactorily with the calculated value of 1.39 g/cm^3 based on two formula units of $C_{22}H_{25}N_2O_4Br$ in a unit cell. The observed systematic absence of $\{0k0\}$ for $k = 2n$ indicates probable space groups $P2_1/m$ and $P2_1$.

A set of three-dimensional counter data collected from a tiny crystal of the bromo derivative was used to compute a three-dimensional Patterson function. Atomic coordinates for the bromine atoms, obtained from an interpretation of the Patterson map, were used in calculation of Fourier syntheses, from which all nonhydrogen light atoms have been located. The refinement of the structure is in progress. The chloro derivative is shown from Weissenberg and precession photographs to be isomorphous with the bromo derivative.

Reference

[1] The samples of both derivatives were kindly furnished to us by Dr. D. G. Doherty of this Division.

30.18 AN ANALYTICAL METHOD FOR EQUATORIAL-PLANE DIFFRACTOMETRY

J. R. Einstein

A graphical and analytical method has been devised for the analysis of intensity measurements in x-ray diffraction experiments with a counter instrument utilizing equatorial-plane geometry. The method facilitates the calculation of the dependence of such measurements on the size, shape, and mosaicity of the crystal; the brilliance distribution of the source; the takeoff angle; the spectral composition of the radiation; the size of the detector aperture; the measurement technique (scan type and scan parameters, or time period of a stationary measurement); and the proximity of neighboring reflections. Crystal size and shape are, for the first time, treated in a general manner. The rest of the treatment represents a recasting of the analysis, as previously worked out by other authors, in a considerably simplified form. The method is of particular usefulness for crystals having large unit cells, for which resolution of neighboring reflections may be difficult.

30.19 ENERGY TRANSFER IN OLIGOTYROSINE COMPOUNDS – FLUORESCENCE TITRATIONS

J. A. Knopp J. W. Longworth

The initial observations on poly-L-tyrosine, where the intensity of the fluorescence of neutral tyrosyl residues was compared with the percent change of neutral to ionized tyrosyl residues, indicated that the fluorescence emission decreased much more rapidly than did the neutral tyrosyl residue concentration. These observations were used to support the concept of energy transfer in poly-L-tyrosine. This concept implied that a similar effect should be observed in oligotyrosine compounds.

We have compared the fluorescence intensity of the neutral tyrosyl residues with the percent of ionization of these residues in a series of tyrosine compounds containing one, two, three, and six residues per molecule. For the compound with one tyrosyl residue, *N*-acetyl-L-tyrosine amide, the fluorescence emission was linearly related to the percent of ionization. For all other compounds, the fluorescence emission was a nonlinear function of percent of ionization. Therefore the concept of efficient energy transfer in tyrosine compounds is confirmed.

To determine the extent of transfer, it is necessary to compare the distribution of molecular species, for example, neutral or singly ionized, with the fluorescence intensity. If the reasonable assumption is made, as we did, that the probability of a residue being ionized is equal to the fraction ionization I of the residues, then the distribution of molecules is given by the terms of the binomial series. That is, the neutral, singly ionized, doubly ionized, etc., are given by

$$(1-I)^n, \quad nI(1-I)^{n-1}, \quad \frac{n(n-1)}{2}I^2(1-I)^{n-2},$$

etc., where n is the number of residues per molecule. In the case where the ionization of one residue per molecule quenches all the other residues by energy transfer, then the fluorescence emission is only due to the neutral species, and the fluorescence intensity is proportional to $(1 - I)^n$.

We compared the experimental values with the function $(1 - I)^n$ and found that the agreement was within experimental error for all compounds except the dityrosine compounds. For the latter compounds, the differences between the experimental values and $(1 - I)^2$ were slightly greater than the experimental error. This discrepancy was interpreted as an electrostatic interaction between residues which makes the second ionization a little more difficult than expected. We conclude therefore that electronic energy transfer between neutral and ionized tyrosyl residues is 100% effective in oligotyrosine compounds up to and including hexa-L-tyrosine.

30.20 CALCULATION OF RATES OF ENERGY TRANSFER IN POLY-L-TYROSINE

J. J. ten Bosch[1] J. A. Knopp[1]

Energy transfer in poly-L-tyrosine has been demonstrated previously at both 298°K[2] and 77°K.[3,4] It appeared that ionized tyrosyl (tyrosinate) residues act as efficient energy scavengers. In order to determine whether or not tyrosyl-tyrosyl transfers preceding tyrosyl-tyrosinate ones play an important role, calculations of transfer rates were performed. Absorption and fluorescence spectra obtained previously in this laboratory[2,3] were evaluated to determine the overlap integrals corresponding to both types of transfer. Extinction coefficients and quantum yields were determined experimentally, both at 298 and 77°K. Together with information on the molecular structure found in the literature,[5] indicating a left-handed α-helical conformation, these values were applied to the theoretical formulas given by Förster's very weak coupling case of energy transfer.

It could be shown that the theory is applicable to the polytyrosine case, which means that an energy quantum is not delocalized over the polymer but that the energy may make jumps over the residue chain. The role of tyrosyl-tyrosinate transfers in the absence of tyrosyl-tyrosyl transfers could be visualized simply by calculating the probabilities that energy present in a certain residue is transferred to the tyrosinate residue. Thus a size was found for a simplified trap defined so that this probability equals 1 inside and 0 outside the trap. To determine the influence of tyrosyl-tyrosyl transfers on this picture, a complete calculation of the probability of the presence of the energy on each residue was made as a function of time and initial position of the energy. One trap was included in a 100-residue-long polymer, and the resulting set of differential equations, involving all transfer rates larger than 10^{-3} transfer per fluorescence lifetime, was solved on a CDC 1604 or IBM 360 computer. The resulting extended trap sizes and other results are shown in Table 30.20.1.

It can be seen that the calculated trap sizes are larger than the observed ones. This may be due to a polymer structure that is less ordered than the assumed structure. We believe, however, that the agreement is certainly good enough to allow the conclusion that the theory describes the actual energy transfer process.

Table 30.20.1. Trap Sizes

Temperature (°K)	When Only Direct Tyrosyl-Tyrosinate Transfers Are Considered	Both Types of Transfers	Observed
77	15.9	~27	20 ± 5^a
298	11.3	28	25 ± 5^b

[a] References 3 and 4.

[b] Reference 3.

References

[1] Biophysics section.

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30.21 ENERGY TRANSFER IN TYROSINE COMPOUNDS – TYROSYL-TYROSYL TRANSFERS

J. A. Knopp[1] J. W. Longworth

Calculations of the rate constants for electronic energy transfer between residues in poly-L-tyrosine demonstrated that tyrosyl-tyrosyl transfers played a role in the overall electronic energy transfer mechanism of this compound. From the magnitude of this contribution, other experiments should reveal its presence. In particular, the limiting polarization of fluorescence,

which is the polarization measured in the absence of molecular rotations, is sensitive to the difference in orientation between the absorber and emitter. Hence, if energy transfer occurs between the absorption and emission of light, this orientation must be different, and the value of polarization will be different.

We determined the limiting polarization of fluorescence resulting from different wavelengths of excitation of a series of tyrosine compounds, including poly-L-tyrosine. Compounds containing more than one tyrosyl residue per molecule exhibited a lower value of polarization than that observed for *N*-acetyl-L-tyrosine amide, which was used as the control. Therefore this is qualitative evidence for the presence of tyrosyl-tyrosyl energy transfers.

As the energy transfer rates were calculated for poly-L-tyrosine, it was possible to calculate the expected value of polarization for poly-L-tyrosine assuming a rigid left-handed α-helical conformation. It was also possible to calculate values for hexa-L-tyrosine and tri-L-tyrosine assuming the same helical conformation and a random conformation. These values are shown in Table 30.21.1 along with the appropriate experimental values. The agreement between the experimental and calculated values is not as good as might be expected. We feel that these differences between the experimental and calculated values reflect the lack of accurate information concerning the conformations of these molecules. We believe, furthermore, that the agreement is sufficient to allow the conclusion that the Förster's very weak coupling case applies to energy transfer between tyrosyl residues and that this type of transfer must be considered in developing the entire energy degradation scheme of tyrosine-containing compounds.

Table 30.21.1. Polarization of Fluorescence with 285-nm Excitation of Tyrosine Compounds Dissolved in Ethylene Glycol at 210°K

Compound	Observed Polarization	Calculated Polarization Assuming:	
		α-Helix	Random
N-Acetyl-L-tyrosine	0.380 ± 0.005		
Tri-L-tyrosine	0.198 ± 0.005		0.137
Hexa-L-tyrosine	0.126 ± 0.005	0.143	0.070
Poly-L-tyrosine	0.109 ± 0.005	0.129	0.00

Reference

[1] Biophysics section.

30.22 ENERGY TRANSFER IN OLIGOTYROSINES – LUMINESCENCE TITRATIONS

Maria Del Carmen Battista J. W. Longworth

Fluorimetric titrations of L-hexatyrosine at 300°K have demonstrated that a single ionized tyrosine residue is sufficient to completely quench the neutral residues by an intramolecular energy transfer. We wished to extend this study to low temperatures to eliminate the possibility of excited-state protolysis, and to be able to measure both singlet and triplet state behavior in the same molecule. Lowering the temperature alters the fractional ionization; so it is necessary to determine the fractional ionization I directly at the temperature of the luminescence measurements. This was accomplished by measuring the absorption spectra of the samples in quartz ESR tubes mounted in a Dewar and placed in the microfocusing attachment of the Perkin-Elmer 450 absorption spectrometer. The fluorescence of the neutral residues followed the dependence $(1 - I)^6$ at both 220 and 77°K, confirming the previous titrations at 300°K. Phosphorescence intensities were also determined, and both the neutral and ionized residues followed the expected behavior. These measurements provided definitive evidence for intramolecular electronic energy transfer at the singlet level in hexatyrosine, where a single ionized residue is sufficient to quench the other five neutral residues.

30.23 ENERGY TRANSFER AND EXCITATION SPECTRA

J. W. Longworth Maria Del Carmen Battista

Excitation spectra have been measured to show that energy absorbed by tyrosine is transferred to tryptophan in the dipeptide L-tryptophanyl-L-tyrosine. The luminescence from the dipeptide is identical with the luminescence of tryptophan. The remarks concerning transfer are general, but we will specifically consider only the tyrosine-to-tryptophan transfer. The excitation spectrum of the tryptophan emission of the dipeptide includes a contribution from the tyrosine absorption. If this transfer is not complete, that is, if there is radiationless internal conversion in tyrosine, then the excitation spectrum will not be identical to the absorption spectrum. To test for identity between the two spectra, the excitation spectrum was divided by the absorption spectrum; this quotient, when normalized, reflects the wavelength dependence of the quantum yield of emission. The quantum yield of fluorescence at 298°K of tyrosine and tryptophan was found to be constant, within 3%, from 300 to 240 nm, and with the

dipeptide L-trp-L-tyr, the quantum yield was constant to within 5%. At 280 nm, tyrosine absorbs 18% of the light absorbed by the dipeptide, and at 300 nm it has no appreciable absorption. Hence these measurements can only set an upper bound, and that is that one-third of the tyrosine absorption in the dipeptide is internally converted. Similar quantum yield studies were made with the fluorescence of the protein ribonuclease T_1, and a large dependence on the wavelength of excitation was found for the quantum yield of fluorescence of the single tryptophan of this enzyme. It was possible to account for the variation in yield by a transfer of only one-third of the energy absorbed by the nine tyrosines of the enzyme. Two-thirds of the energy absorbed by tyrosine is internally converted by radiationless transition at the singlet level.

30.24 THE EXCIPLEX NATURE OF ENDONUCLEASE FLUORESCENCE

J. W. Longworth

The endonuclease of *Staph. aureus* (E.C. 3.1.4.7) has a single tryptophan residue, and the fluorescence is dominated by emission from this residue. At 77°K the fluorescence is fine structured, and the intensity maximum is at 317 nm; but at room temperatures, the emission has no fine structure, is red shifted, and the intensity maximum lies at 335 nm. Two explanations for the effect of temperature on the character of the emission have been proposed. Mechanism 1 is that of a complex formation with a neighboring polar group by the excited state (exciplex), and mechanism 2 consists of a reorientation of the solvation shell around the excited state. To distinguish between these two mechanisms the fluorescence of the protein and model systems was measured at various temperatures in glassing solvents. Mechanism 1 is expected to lead to a common intersection point in the fluorescence since only two species are involved, whereas mechanism 2 results in a progressive shift in the intensity maximum to longer wavelengths with increasing temperature. An isoemissive point is found in the fluorescence spectra of endonuclease measured at various temperatures between 150 and 250°K, and this indicates that exciplex formation occurs at the higher temperatures. In contrast, model systems of tryptophan compounds fit the behavior expected for mechanism 2.

30.25 OPTICAL PROPERTIES OF POLYNUCLEOTIDES DENATURED BY ORGANIC SOLVENTS

J. A. Knopp[1] J. W. Longworth
R. O. Rahn[1]

A random coil conformation of polynucleotides [in particular, DNA, tRNA, $d(A\text{-}T)_n$, $d(A\text{-}C)_n$, $d(G\text{-}T)_n$, and $r(A)_n$] has been obtained by dissolving lyophilized fibers into ethylene glycol (EG) that contains 0.1 *M* NaCl. Polymers in this conformation are found to have different luminescent, circular dichroism, and thermal melting behaviors than in their duplex or single-strand stacked conformations (which exist when 5% water is present). Fluorescence at 77°K of the random coil is like that of monomers and unlike the exciplex emission from duplex polymers. Electron spin resonance and phosphorescence measurements have shown that the only triplet is that of thymine for DNA as a duplex or as a stacked single strand, but the random coil has adenine as the predominant triplet with the thymine remaining unchanged. Polyd(A-T) only phosphoresces from thymine in the random coil, whereas a mixture of polyd(A-C) and (G-T) has both an adenine and a thymine phosphorescence. This suggests that adenine and guanine can transfer energy to thymine in a random coil, but that the transfer is only short range, since thymine does not completely quench adenine in DNA.

Reference

[1] Biophysics section.

30.26 ELECTRONIC ENERGY TRANSFER IN POLYNUCLEOTIDES

J. W. Longworth R. O. Rahn[1]
J. A. Knopp[1] Maria Del Carmen Battista

The presence of intramolecular electronic energy transfers in polynucleotides has been frequently proposed and exhaustively calculated, but experimental support is rare and indirect. Electronic energy transfer is suggested by the absence of emission of a suspected donor in the presence of a potential acceptor, but to directly investigate transfer, experiments on sensitization of the acceptor or fluorescence polarization measurements must be performed. We have obtained some

qualitative results which indicate that transfers do occur. As reported in the previous abstract, the random coil of polyd(A-T) phosphoresced only from the T residues, and since the excitation spectrum is similar to the absorption, this result suggests that A transfers to T, which is consistent with the sequence of triplet energies. However, the polymer in basic solutions has emission from both A and T^-, yet the triplet energies indicate that T^- ought to transfer to A. Nevertheless the random ribopolynucleotides $r(A,U)_n$, $r(A,G)_n$ in the random coiled form at neutral and alkaline pH values always phosphoresce from the A residue alone, suggesting transfers to A – a result consistent with the known sequence of triplet levels. Likewise, the mixture of random coiled polymers polyd(G-T) and polyd(A-C) has no G emission, so that T can quench G. Hence A is an effective trap of the triplet energy in polynucleotides and can be quenched by T, as in $(A-T)_n$, but not entirely in the random coil of DNA and tRNA. Phosphorescence excitation of T is similar to the DNA absorption in a DNA duplex and is like the excitation spectrum of A in the random coil. Both observations suggest extensive intramolecular transfers. However, the excitation spectrum of the fluorescence of the random coil is different from that of the phosphorescence. This suggests that there are radiationless processes which compete with transfers at the singlet level, but since the phosphorescence excitation spectrum of one of the emitting residues resembles the absorption, then the simplest interpretation would be that the radiationless process is an intersystem crossing accompanied by triplet transfers. The polymer polyrA, in the single-strand stacked conformation, is an attractive system to apply fluorescence polarization measurements to transfer studies. Theoretical calculations by several groups indicate that within the singlet lifetime there are expected to be transfers at the singlet levels over tens of bases. However, the fluorescence polarization as a function of absorbing wavelength at 220°K in propylene glycol is identical to that found from AMP, and so there cannot be any singlet transfers. Hence, there are qualitative indications of transfers, particularly at the triplet level, and these transfers appear to depend on the polynucleotide conformation and the base sequence.

Reference

[1] Biophysics section.

30.27 LUMINESCENCE OF MIXED tRNA's FROM *ESCHERICHIA COLI*

J. W. Longworth M. P. Stulberg[1]

Native tRNA has a strong phosphorescence contribution from the thiouracil residues when excited at 330 nm. However, when excited at 260 nm, there is a completely different emission, which has no thiouracil component and has a very small quantum yield. The phosphorescence emission resembles that observed with polyrG,[2] while the fluorescence is that of an exciplex and is unlike that found from polyrG. The exciplex emission is absent in the random coiled conformation, and there is now a strong fluorescence, similar to that of the monomers. The phosphorescence is also greatly enhanced and predominantly from the A residues. There is, however, another component, which is short-lived (800 msec) and which lies to longer wavelengths than A, and has no fine structure. This component is preferentially excited by light with wavelengths longer than 290 nm. This emission resembles that previously observed with polyr(A-U), and we provisionally ascribe the emission to U. The thiouracil contribution is unaltered by forming a random coil. The emission from tRNA's at pH 12 is similar to that of the random coil. As a model for tRNA, the random copolymer $r(A,G,C,U)_n$ was investigated, and the phosphorescence was from both A and U; the exciplex fluorescence of the stacked conformation is destroyed and replaced by monomer-like emission in the random coiled conformation.

References

[1] Enzymology section.
[2] J. A. Knopp, personal communication.

30.28 RELATIONSHIP BETWEEN CARCINOGENICITY AND MUTAGENICITY OF METHYLATING COMPOUNDS

H. V. Malling F. J. de Serres

Methyl methanesulfonate (MMS) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) are both methylating compounds and are able to methylate the bases in the DNA. Both compounds are mutagenic in several different organisms. However, whereas MNNG is a strong

systemic carcinogen, MMS has only very limited carcinogenic activity. It is possible that the higher carcinogenic activity of MNNG than MMS is correlated with a particular type of chemical activity, and that by comparing the mutagenicity of MNNG and MMS at the molecular level, this problem can be evaluated.

Forward-Mutation Frequency. – Conidia from a genetically marked balanced two-component heterokaryon of *Neurospora crassa* were treated for 5 hr in a 0.067 *M* phosphate buffer at pH 7.0 with various concentrations of MMS. Forward mutations in the *ad-3* region induced in the heterokaryotic fraction of the conidia were obtained by the direct method. The study of the mutagenicity of MNNG in *Neurospora crassa* has been reported previously. MNNG is much more mutagenic in *Neurospora* than MMS; under similar treatment conditions with respect to pH, temperature, and shaking (4 hr treatment with 25 μM MNNG and 5 hr treatment with 20 m*M* MMS) 1.45×10^{-3} and 3.5×10^{-4} *ad-3* mutations per survivor were induced respectively.

Complementation Pattern. – The most striking difference between the mutagenesis of MNNG and MMS at the molecular level at the present stage of the analysis is in allelic complementation among the *ad-3B* mutants. *Ad-3B* mutants are either noncomplementing or have a polarized or a nonpolarized complementation pattern. Among the MNNG-induced *ad-3B* mutants, 81% had nonpolarized complementation patterns, 1% polarized complementation patterns, and 18% were noncomplementing. Among the MMS-induced *ad-3B* mutants, 39% had nonpolarized complementation patterns, 11% polarized complementation patterns, and 50% were noncomplementing.

When the spectra of complementation patterns induced by MNNG and MMS in *Neurospora* are compared with similar spectra induced by such specific mutagens as nitrous acid (NA), hydroxylamine (HA), and *O*-methylhydroxylamine (OMHA) (Table 30.28.1), tentative conclusions can be reached about the mutation mechanism of MMS and MNNG. The genetic alterations in the NA- and HA-induced mutants have been identified by tests for revertibility after treatment with specific mutagens. We found that the predominant portion of the nitrous-acid-induced base-pair transitions have GC at the mutant site, whereas most of the HA-induced mutants have AT at the mutant site. HA is an extremely specific reagent for cytosine in the DNA[1] and induces predominantly base-pair transitions from GC to AT.

It is easy to see that the spectra of the complementation patterns among mutants induced by these two mutagens reflect the genetic alteration identified at the mutant site. From what is known about the genetic code, we know that mutants induced by a GC → AT transition can be nonsense mutations, whereas mutants induced by an AT → GC transition will never give rise to a nonsense codon.

Mutants with a nonsense codon will code either for a fraction of the polypeptide chain or for no polypeptide at all. Therefore these mutants will have a polarized complementation pattern or be noncomplementing. Mutants induced by an AT → GC transition should be predominantly missense mutations, and therefore code for a complete polypeptide with only a single erroneous amino acid. The great majority of these mutants will have a nonpolarized complementation pattern, like the mutants induced by NA.

As we can see in Table 30.28.1, the spectrum of complementation patterns for a certain mutagen seems to have a diagnostic value for the determination of the genetic alteration induced by this mutagen at the molecular level. If we compare the spectrum of complementation patterns among MNNG-induced *ad-3B* mutants with the spectrum among mutants induced by NA, HA, or OMHA, which is more specific in its mutagenic action than HA, we can arrive at the tentative conclusion that mutants induced by MNNG preferentially have GC at the mutant site. The predominant mechanism of mutation induction by MNNG

Table 30.28.1. Percentage of Different Types of Complementation Patterns Among *ad-3B* Mutants Induced by Five Different Mutagens

Abbreviations:
NP = *ad-3B* mutants with nonpolarized complementation pattern
P = *ad-3B* mutants with polarized complementation pattern
NC = noncomplementing *ad-3B* mutants
MNNG = *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
NA = nitrous acid
HA = hydroxylamine
OMHA = *O*-methylhydroxylamine
MMS = methyl methanesulfonate

Mutagen	Complementation Patterns		
	NP	P	NC
MNNG	81	1	18
NA[a]	75	7	18
HA	52	12	36
OMHA	45	12	43
MMS	37	13	50

[a]Complementation pattern among the NA-induced mutants. With a base-pair transition at the mutant site.

appears to be base-pair transition from AT to GC (this point is currently being investigated by studying the revertibility of MNNG-induced mutants after treatment with specific mutagens).

Noncomplementing mutants and mutants with polarized complementation patterns can arise from a broad spectrum of genetic alterations[2] which result in the production of polypeptide fragments or nonfunctional gene products. When the complementation spectrum of MMS-induced mutants is compared with the spectra of HA-induced and NA-induced mutants (Table 30.28.1), it resembles that of HA-induced mutants. By analogy, this suggests that most MMS-induced mutants may have AT at the mutant site, a result in good agreement with the observation that MMS reacts predominantly with guanine in the DNA. It is likely, however, that the MMS-induced mutants are composed of a wide variety of genetic alterations, similar to the EMS-induced mutants in *Neurospora.*[3]

MMS seems, therefore, to give rise to a higher frequency of mutants which specify a nonfunctional gene product than does MNNG. Compounds which induce mutations that block normal gene function completely may be more efficient at inactivation of the cell, whereas compounds which alter normal gene function are more likely to alter normal biosynthesis and metabolism. On the basis of these and similar data we have formulated the following tentative working hypothesis: that compounds which induce a high percentage of mutants in which the specific gene product has an altered function are strong carcinogens, whereas compounds which induce a high percentage of mutants in which the specific gene product is nonfunctional will only have a limited carcinogenic activity.

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30.29 GENETIC ANALYSIS OF TRIETHYLENEMELAMINE-INDUCED PURPLE MUTANTS (*ad-3*) in *NEUROSPORA CRASSA*

H. V. Malling F. J. de Serres

Triethylenemelamine (TEM) has a strong mutagenic effect in many different types of organisms (mammals, plants, microorganisms, etc.). TEM is a trifunctional alkylating agent which can react in vitro with DNA and is able to cross-link the two helixes in the DNA molecule. Of the DNA components there is some indication that TEM reacts predominantly with thymine.[1] TEM is an important antineoplastic compound but has also carcinogenic properties. It is able to induce both chromosome breaks and point mutations, but in most systems these mutational events cannot be scored simultaneously. Furthermore, TEM is one of the few chemical mutagens whose mutagenic action has been studied in the specific locus system in mice.[2]

We feel that it is important to study the mutagenic effect of TEM in a eukaryotic system, where both chromosome damage and point mutations can be detected and the genetic alterations identified at the molecular level.

In the dikaryon system of *Neurospora crassa,*[3] the following biological parameters can be studied: (1) nuclear and cytoplasmic inactivation, (2) frequency of recessive lethal mutations over the entire genome, (3) frequency of chromosomal deletions in the *ad-3* region, (4) frequency of point mutations in the two structural genes *ad-3A* and *ad-3B*, and (5) identification of the genetic alteration at the molecular level.

Conidia from a genetically balanced heterokaryon were treated for various lengths of time (20 to 170 min) with 0.01 *M* TEM dissolved in a phosphate buffer adjusted to pH 7.0. In this experiment we found the following results: (The description of the relation between mutation frequencies and the length of treatment time refers to a log-log plot. The values of the slopes are therefore equal to the power of the treatment time in this correlation.)

1. A much lower survival of the heterokaryotic fraction of the conidia compared with survival of the total population of conidia for the various times of treatment by TEM. This indicates that a part of the inactivation of the conidia by TEM is due to inactivation of the nuclei.
2. The mutation frequencies of the point mutations in *ad-3A* and *ad-3B* increase with a slope greater than 1 (1.35). The chromosome deletions ($ad\text{-}3^{IR}$) in the adenine region increase with a slope greater than 2. Since the point mutations only increased with a slope of 1.35, this indicates that the chromosome deletions in the *ad-3* region most likely are the results of two independent reactions of TEM with the chromosomes. As far as we are aware, TEM is the first chemical which has been shown to induce chromosome breaks in fungi.
3. Genetic analysis (Table 30.29.1) of the mutants by means of heterokaryon tests for complementation

Table 30.29.1. Frequency of the Different Types of Mutations Induced by TEM in the *ad-3* Region of *Neurospora crassa*

ad-3A: includes all point mutations in the *ad-3A* locus, also including the *ad-3A* mutants which have additional damages elsewhere in the genome.

ad-3B: includes all point mutations in the *ad-3B* locus, also including the *ad-3B* mutants which have additional damage elsewhere in the genome.

ad-3R+RL: includes all point mutations in either the *ad-3A* or the *ad-3B* locus which have an additional damage elsewhere in the genome.

ad-3 ^{IR}: all irreparable mutations covering either the *ad-3A* or the *ad-3B* locus or both.

Treatment (min)	Mutation Frequency per 10^6 Survivors				
	ad-3A	*ad-3B*	*ad-3R+RL*	*ad-3*^{IR}	Total Forward-Mutation Frequency (directly determined)
0	0.3	0.3	0	0	0.5
20	9.8	11.5	0.5	0.8	22.4
40	24.7	23.1	3.1	1.2	49.3
80	75.8	53.7	18.2	1.0	132.3
120	106.3	109.7	57.0	6.9	227.0
170	185.6	202.9	133.0	32.9	426.8

showed that the frequencies of *ad-3A* and *ad-3B* mutants increase with increasing length of treatment time and then with similar slopes which are both greater than 1. The frequencies of *ad-3B* mutants with a polarized complementation pattern and non-complementing *ad-3B* mutants increased with powers of 1.67 and 1.56 of the treatment time, respectively, whereas mutants with nonpolarized complementation pattern increased in direct proportion to the length of treatment time. The frequency of the three classes of mutants within the *ad-3B* locus varies therefore with the treatment time. After 20 min treatment time the percent of non-polarized, polarized, and noncomplementing mutants were, respectively, 65, 4, and 31; after 170 min treatment the percent of nonpolarized mutants dropped to 43, whereas the percent of polarized mutants increased to 13 and the percent of noncomplementing mutants increased to 44. From these data we can conclude that short treatments of TEM mainly give rise to point mutations and that at least more than half of these are due to base-pair substitutions, whereas mutants induced after longer treatments are derived from more drastic genetic alterations.

TEM possesses carcinogenic activities as well as antineoplastic activities. In a previous study of mutagenesis of methylating compounds with respectively potent and weak carcinogenic activity, we formulated the following working hypothesis: that compounds which induce a high percentage of mutants in which the specific gene product has an altered function are strong carcinogens, whereas compounds which induce a high percentage of mutants in which the specific gene product is nonfunctional will only have a limited carcinogenic activity.

The present data indicated therefore that long-term treatments with lower concentrations of TEM should be more carcinogenic and less antineoplastic and vice versa for short treatments with higher concentrations of TEM.

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30.30 PHOTODYNAMIC EFFECT OF POLYCYCLIC HYDROCARBONS ON *NEUROSPORA CRASSA* AND CHINESE HAMSTER CELLS

H. V. Malling E. H. Y. Chu F. J. de Serres

In a previous report[1] the photodynamic effect of the two similar polycyclic hydrocarbons 1,2-benzopyrene (1,2-BP) and 3,4-benzopyrene (3,4-BP) was studied in two different systems: (1) conidia from a heterokaryon between genetically marked strains of *Neurospora* and (2) Chinese hamster cells in tissue culture. Of these two compounds, only 3,4-BP is carcinogenic and was also the only one which showed a photodynamic effect. Brookes and Lawley[2] have found a good correlation between the reactivity of a series of polycyclic hydrocarbons with DNA in the mouse skin and Iball carcinogenic index for these compounds. In the present study, we have used the same series of polycyclic hydrocarbons, namely: 1,2-BP, 3,4-BP, 9,10-dimethyl-1,2-benzanthracene (9,10-DMBA), 20-methylcholanthrene (20-MC), 1,2,5,6-dibenzanthracene (1,2,5,6-DBA), and 1,2,3,4-dibenzanthracene (1,2,3,4-DBA). None of these compounds had any toxic effect in the dark on *Neurospora* conidia. On hamster cells, only 9,10-DMBA showed a toxic effect in the dark.

Two of the three strongest carcinogens of this series of polycyclic hydrocarbons, 3,4-BP and 9,10-DMBA, showed a strong photodynamic effect in both *Neurospora* and hamster cells; the third strong carcinogen, 20-MC, did not show a pronounced photodynamic effect in either system. The compounds with weak or no carcinogenic activity (1,2-BP, 1,2,3,4-DBA, and 1,2,5,6-DBA) had no toxic effect on *Neurospora* conidia or hamster cells when the cells were posttreated with black light.

By plating *Neurospora* conidia from the heterokaryon on selective substrate, it is possible to distinguish between inactivation of the conidia due to inactivation of the nuclei or inactivation of the cytoplasm. In all cases where we found a photodynamic effect of the polycyclic hydrocarbons, the inactivation of the conidia seems to be due to inactivation of the cytoplasm.

For this series of polycyclic hydrocarbons, there seems to be a reasonably good correlation between the photodynamic effect and the carcinogenic activity of the compounds. Other compounds which are not carcinogenic, such as acridines, have shown a photodynamic effect in several biological systems. Both in *Neurospora* and Chinese hamster cells, we can study a wide spectrum of genetic effects. It seems likely that by contrasting the genetic alterations induced by a combined treatment of black light and (1) carcinogenic polycyclic hydrocarbons with (2) noncarcinogenic acridines, we would be able to predict which part of the photodynamic activity is related to the induction of cancer.

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30.31 INDUCTION OF TRANSPLANTABLE RETICULUM CELL SARCOMA IN BALB/c MICE FOLLOWING REPEATED INJECTIONS OF SHEEP ERYTHROCYTES

R. L. Tyndall J. A. Otten[1] A. C. Upton

Introduction. – Immunological studies have suggested that repeated injections of antigen might be leukemogenic. To test this possibility, thirty 12-week-old male BALB/c and eleven female AKR mice were injected intraperitoneally twice weekly with 1 ml of a 10% washed sheep red blood cell (SRBC) suspension. These injections were continued for a 12-week period in the BALB/c mice, during which time 11 of the mice died from causes unknown.

Results and Discussion. – Toward the end of treatment the surviving BALB/c mice showed signs of listlessness, ruffling of fur, and diarrhea. After cessation of treatment (12 weeks), all remaining 19 BALB/c mice showed various degrees of splenomegaly as detected by palpation. One of five animals autopsied at this time was diagnosed as leukemic on histological examination; the other four showed splenic hyperplasia. In the ensuing eight weeks, ten additional mice were autopsied, nine of which were diagnosed as leukemic and one as having splenic hyperplasia. The four remaining mice showed splenic regression shortly after cessation of treatment, but all four subsequently died from leukemia 20 to 30 weeks after SRBC treatment (Table 30.31.1).

The leukemias were classified as reticulum cell sarcomas (RCS), and all of 20 newborn BALB/c mice inoculated intraperitoneally with approximately 5×10^7 nucleated spleen cells from one of the leukemic animals developed leukemia within four weeks following injection. Malignant cells from a second donor showed similar results on transplantation. Injection of

Table 30.31.1. Effect of Repeated Inoculations of Sheep Erythrocytes in BALB/c and AKR Mice

Experiment	Strain	Treatment	Incidence of Effects				
			Morbidity[a] by End of Treatment	Splenomegaly[b] by End of Treatment	Leukemia[c]		
					Within 11 to 13 Weeks After Start of Treatment	Within 13 to 20 Weeks After Start of Treatment	Within 20 to 30 Weeks After Start of Treatment
1	BALB/c	SRBC	19/19	19/19	1/5	9/10	4/4
1	AKR	SRBC	0/12	0/12	0/12	0/12	0/12

[a] Morbidity characterized by listlessness, ruffling of fur, diarrhea; particularly pronounced following inoculation.

[b] Splenomegaly determined by abdominal palpation.

[c] Number of leukemias (majority histologically confirmed)/total number of mice necropsied.

10^5 to 10^7 x-irradiated (15,000 r) pooled malignant cells from two mice with such passaged reticulum cell sarcoma was found to transmit the disease to all of 15 recipients. The lack of spontaneous leukemia in BALB/c mice within the first year of life, observed in over 200 BALB/c mice in our animal facility as well as in other colonies, strongly indicates that the leukemias in mice following treatment with SRBC resulted from the SRBC treatment per se.

To what degree breakdown of the antigen-trapping mechanism, immunological paralysis, increased production of immunoblasts, and/or activation of latent leukemia virus(es) are involved in the induction of RCS following SRBC treatment in BALB/c mice remains to be determined. The passage of these RCS with cells irradiated with 15,000 r, however, may be indicative of a viral etiology. Also to be determined is the reproducibility of the effect of SRBC treatment of BALB/c in other mouse strains. The lack of any overt symptoms thus far in the similarly treated female AKR mice implies that the reaction may be influenced by genetic, hormonal, or other environmental factors (Table 30.31.1). Also to be explored are the effects of antigens other than SRBC and of the trauma of repeated inoculations. It is hoped that further studies presently under way will answer many of these questions.

Reference

[1] Pathology and Physiology section.

30.32 ALTERED PROTEIN AND ESTERASE PROFILES OF RETICULUM CELL SARCOMAS RESULTING FROM RAUSCHER LEUKEMIA VIRUS INFECTION

R. L. Tyndall R. C. Allen[1]
Ernestine Teeter Dorothy J. Moore[1]

Introduction. – A previous report described the increased esterase isozyme activity associated with infection of both cultured and malignant spleen cells infected with the Rauscher leukemia virus.[2] The present study was undertaken to study both esterase isozyme and protein profiles of sera, spleen, and thymus tissue from leukemic BALB/c mice infected with Rauscher leukemia virus (RLV).

Results and Discussion. – Overtly leukemic and comparably aged normal BALB/c mice were bled via heart puncture, perfused with normal saline, and their tissues of interest removed and frozen at –70°C. When thawed, the tissues were homogenized and extracted in Ringer's solution, the cell debris was removed by centrifugation, and the soluble protein supernatants were electrophoresed on acrylamide gel. Esterase activity was determined using α-naphthyl butyrate as substrate and Fast Blue RR salt as the diazo coupling agent. Protein patterns were determined by staining with Aniline Blue Black.

Extracts of leukemic spleen tissue (reticulum cell sarcoma) showed a depression of prealbumin esterase and a concomitant intensification of two particular isozyme bands, when compared with normal spleen extracts. The degree of intensification in the leukemic extracts appears to be related to the degree of malignant infiltration as determined by histologic examination of sample tissue from each spleen. Protein profiles of leukemic spleen extracts revealed the acquisition of proteins with electrophoretic mobilities identical to serum albumin, transferrin, and other serum proteins, with a concomitant loss of protein bands present in normal spleen extracts. Nonmalignant thymus tissue from leukemic animals generally showed either intensification of several esterase isozymes or a generalized diminution of esterase activity. Thymus protein patterns were not apparently altered. No alterations of either esterase or protein profiles were apparent in serum from leukemic animals. Analysis of extracts from fetal or newborn spleen pools indicated a similarity between these esterase profiles and those of leukemic spleen extracts. No obvious differences, however, were observed between the protein profiles of a control uninfected (JLS V9) and those of an RLV-infected cell culture (JLS V10), neither of which showed any similarity with the sera protein pattern.

The results of these experiments indicate that the major alterations in esterase and protein profiles are associated with the malignant reticulum cell sarcoma (RCS) per se and with some as yet unexplained esterase alterations in thymus tissue. The lack of protein profile changes in the RLV-infected JLS V10 cell culture and the dissimilarity between such protein profiles and serum protein patterns indicates that the altered protein patterns in RLV-infected RCS tissue are probably a reflection of the malignant state rather than a reflection of virus production. The significance of apparent serum protein production in RCS tissue and the similarity in esterase profiles between RCS and fetal spleen tissue remain to be determined. Experiments are presently under way to determine if the analogy between fetal spleen and RCS tissue extends to other isozyme systems and to the antigenicity of the two tissues.

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[1] Pathology and Physiology section.

[2] R. L. Tyndall *et al., Biol. Div. Ann. Progr. Rept. Dec. 31, 1967,* ORNL-4240, p. 319.

30.33 SERINE AND ASPARAGINE REQUIREMENTS IN NORMAL AND LEUKEMIC HUMAN BLOOD CELLS

James D. Regan Susumu Takeda[2]
Helen Vodopick[1] W. H. Lee
F. M. Faulcon[3]

It is known that some animal leukemias have a requirement for asparagine because they are sensitive to, and are repressed or even cured by, injections of asparaginase.[4] Asparaginase has also been used in the treatment of human leukemia, with the tacit assumption that there is no asparagine requirement in *normal* human bone marrow.[5] In fact there exists a paucity of data on the asparagine requirements of normal marrow and no data at all on possible requirements for the other six in vitro nonessential[6] amino acids. If asparagine has significance in this regard, then it is clear that any of the other nonessential amino acids could be equally important since asparagine is noteworthy only in that it is a member of this group.

Accordingly, we undertook the investigation of the nonessential amino acids in human granulocytic leukemia (both the acute and chronic forms) and in normal human bone marrow cells. Our experiments were mainly incorporation experiments with radioactive macromolecular precursors, experiments to determine the amount of synthesis of certain nonessential amino acids in these cells, and some experiments on the activity of enzymes involved in the biosynthesis of certain nonessential amino acids.

Figure 30.33.1 is a summary histogram of our incorporation experiments. These data are applicable to both leukemic cells and cells from normal human marrow since both cell types behave essentially the same in these experiments. The data show that while asparagine is partially required by leukemic cells it is also required by normal bone marrow. However, another nonessential amino acid, serine, is required much more severely by both cell types than is asparagine.

Table 30.33.1 compares the ability of WI-38 normal human diploid fibroblasts (a serine-independent cell type) to synthesize ^{14}C-serine from ^{14}C-glucose with this capability in leukemic cells and normal human bone marrow. WI-38 cells were highly capable of serine synthesis, while bone marrow and leukemia cells were essentially incapable of this synthesis.

We have also preliminarily examined 3-phosphoglycerate dehydrogenase activity (one of the principal enzymes in serine biosynthesis[7]) in serine-competent human cells, such as HeLa and WI-38, and in serine-dependent leukemic cells. The serine-competent cells had high activity for 3-phosphoglycerate dehydrogenase, while the leukemic cells had little or no such activity.

These results seem to indicate that a partial asparagine requirement and a more severe serine requirement are present in normal human bone marrow and in cells derived from bone marrow such as granulocytic leukemia cells.

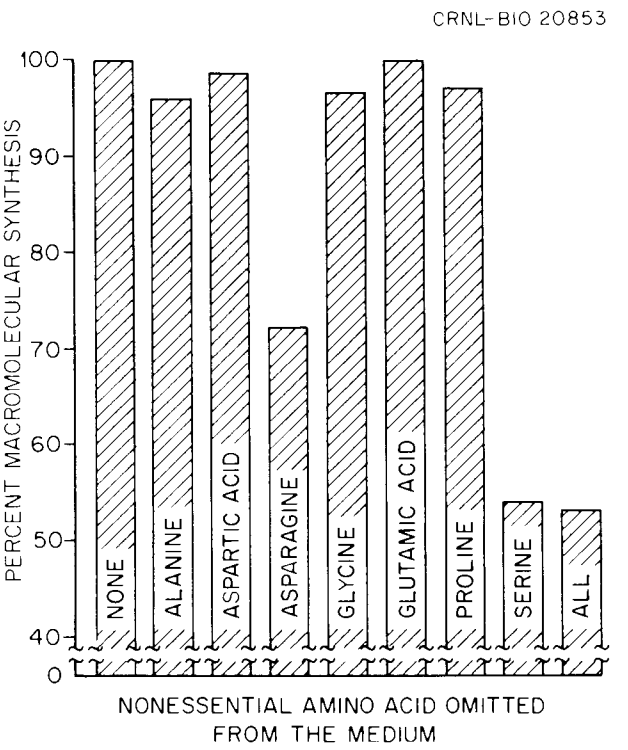

Fig. 30.33.1. The Effect on Macromolecular Synthesis of Omitting Different Nonessential Amino Acids from the Culture Media in an in Vitro Test to Assay for Nutritional Requirements in Leukemic and Normal Human Cells. Syntheses of biological macromolecules (nucleic acids and protein) were determined by incorporation of radioactive precursors. Data are average results for a number of different experiments with leukemic cells and cells of normal human bone marrow.

Table 30.33.1. Radioactivity in Total Cellular Serine After 48 hr Incubation in ^{14}C-Glucose

Cells	Counts per Minute per Micromole of Serine
WI-38 human diploid fibroblasts	317,700
Leukemic cells	2,100
Normal bone marrow cells	1,395

Thus it appears possible that a rationale may exist for leukemia therapy involving the enzyme serine dehydratase or serine antimetabolites, providing that the growth and metabolic rates of the leukemic cells are sufficiently elevated above those of the normal cells.

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[1] Medical Division, Oak Ridge Associated Universities.
[2] Biophysics section.
[3] Mammalian Cytogenetics section.
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[6] H. Eagle, *Science* **130**, 432 (1959).
[7] L. Pizer, *J. Biol. Chem.* **239**, 4219 (1964).

30.34 A SERINE REQUIREMENT IN HUMAN LYMPHOBLASTOID ESTABLISHED CELL LINES

James D. Regan W. H. Lee F. M. Faulcon[1]

We have recently presented evidence for a serine requirement in human granulocytic leukemia cells and in cells from normal human bone marrow.[2] We have also examined a number of established cell lines of human lymphoblastoid origin for their amino acid requirements. These cell lines were developed from normal subjects and from patients with various blood diseases such as infectious mononucleosis, lymphosarcoma, or chronic granulocytic leukemia.[3] All the cell lines of the lymphoblastoid type thus far examined exhibited a serine requirement. In contrast, heteroploid monolayer cells such as HeLa, diploid fibroblasts (WI-38), and a quasi-diploid squamous cell carcinoma cell line (RPMI 2650) were all serine independent.

A highly consistent and selective marker such as serine dependence can be quite useful in somatic cell genetics. Thus we are employing this marker in experiments to determine (1) the rate of spontaneous and induced reversion to serine independence and (2) whether serine dependence behaves as a dominant or recessive character upon hybridization with a serine-independent cell.

References

[1] Mammalian Cytogenetics section.
[2] James D. Regan *et al.*, *Science*, in press (1969).
[3] The lymphoblastoid cell lines described above were kindly furnished to us by Dr. Philip Glade, of Mt. Sinai School of Medicine, New York City, and by Dr. George E. Moore, of Roswell Park Memorial Institute, Buffalo.

30.35 INHALATION CARCINOGENESIS

D. G. Doherty M. G. Hanna, Jr.
J. O. Brick Paul Nettesheim
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The first chamber exposure phase of the role of air pollutants on the induction of pulmonary tumors in mice that have been pretreated with a radiation, viral, or combined insult is nearly complete. Eight of the groups have terminated, and the remaining six are down to small numbers of animals. The treatments and total exposure times are given in Table 30.35.1. All animals to date have remained free from pseudomonas, salmonella, internal and external parasites, and the seven murine viruses originally typed, and there has been a minimal amount of respiratory disease. The median death time of the animals in the experiment has been estimated and is presented in Table 30.35.2. The stress of living on wire or daily in the chambers is reflected in the average median death times of those with no exposure to chemicals, for example, males in pans 29 months, wire 25 months, chambers 20 months; females in pans 26 months, wire 21 months, chambers 23 months. In the males at least this can be directly correlated with the incidence of genitourinary disease, which is low in those living in pans, fourfold higher in the wire group, and sevenfold higher in the chamber group. There is an anomalous increase in median death time in six of the irradiated female groups.

The data from this study have been programmed for automatic record storage, retrieval, and analysis, and at present an examination of the various interactions between the experimental groups is in process. The age-specific incidence of lung adenomas for the first three chambers is given in Table 30.35.3. There is a consistent 100-day earlier incidence in lung adenomas in the smog-treated groups over the control and the chromic oxide group. With regard to chemical treatment the gasoline smog is also the most effective agent, as may be seen from Table 30.35.4. The total number of tumors is 15 in the washed air chamber, 16 in the chromic oxide group, and 30 in the smog-treated group. An analysis of the additional control groups with no exposure to chemicals awaits their termination.

Table 30.35.1.

A. Chamber Operations Through December 31, 1968

Chamber No.	Pretreatment	Chemical	Exposure (hr)	Completed
I	One-half irradiated	0	3133	May 1968
II	One-half irradiated	Cr_2O_3		March 1968
		52 ppm	2723	
		27 ppm	363	
III	One-half irradiated	Ozone, 1 ppm; gasoline, 30 ppm	3093	April 1968
IV	One-half irradiated – all virus	Ozone, 1 ppm; gasoline, 30 ppm	2959	May 1968
V	One-half irradiated – all virus	Cr_2O_3		June 1968
		53 ppm	2540	
		27 ppm	363	
VI	One-half irradiated – all virus	0	2813	July 1968
VII[a]	0	45 pans	0	
VIII[a]	Irradiated	45 pans	0	Dec. 1968
IX[a]	Virus	45 pans	0	
X[a]	Irradiated – all virus	45 pans	0	
XI[b]	One-half irradiated	45 pans	0	
XII[b]	One-half irradiated – all virus	45 pans	0	Nov. 1968
XIII	One-half irradiated – all virus	$CaCrO_4 \cdot H_2O$, 35 ppm	2065	
XIV	One-half irradiated	$CaCrO_4 \cdot H_2O$, 35 ppm	2171	

[a]No exposure in the chambers – kept in pans to compare with chambers I and VI controls.

[b]No exposure in chambers – kept in wire cages to compare with chambers I, VI, VII, VIII, IX, and X controls.

B. Mouse Disposition Through December 31, 1968

Total initial number of mice (all chambers to date)	7547
Sacrificed at chamber startup (pathology, etc.)	558
Total to chambers	6989
Sacrificed for 6, 12, & 18 month examinations	615
Sacrificed for electron microscopy	35
Terminal sacrifice (pathology, etc.)[c]	596
Previously found dead or sacrificed	4965
Deaths this month	122
Total deaths, all causes	6333

[c]Last 15 of each chamber category (♂ – irradiated or nonirradiated; ♀ – irradiated or nonirradiated) are sacrificed.

Table 30.35.2. Median Death Time (Months) of C57Bl/6 Mice

Chamber	Viral Pretreatment	Male		Female	
		0	100 r	0	100 r
I	0	22	20	25	25
II	0	22	20	16	18
III	0	25	22	20	25
IV	Virus	20	20	25	25
V	Virus	17	18	22	24
VI	Virus	17	20	18	25
VII	0	31		26	
VIII	0		30		26
IX	Virus	29		26	
X	Virus		28		26
XI	0	26	26	19	24
XII	Virus	25	24	20	22
XIII	Virus	(29)	21	20	19
XIV	0	(30)	22	22	20

Table 30.35.3. Lung Adenoma and Adenocarcinoma Incidence

	Time (days)						Summary
	400	500	600	700	800	900+	
Washed Air Chamber Mice							
Number with tumors	0	0	1	2	9	3	Total number of lung tumors, 15
Number of deaths	72	48	56	60	84	130	Total of animals, 450
Percentage	0	0	1.8	3.3	10.7	2.3	Total percentage, 3.3
Smog Chamber Mice							
Number with tumors	0	0	3	6	10	11	Total number of lung tumors, 30
Number of deaths	76	48	60	64	96	106	Total of animals, 450
Percentage	0	0	5.0	8.8	11.4	8.2	Total percentage, 6.7
Chromate Chamber Mice							
Number with tumors	0	1	2	2	2	9	Total number of lung tumors, 16
Number of deaths	52	48	60	68	88	134	Total number of animals, 450
Percentage	0	2.1	3.3	3.1	2.1	8.5	Total percentage, 3.6

Table 30.35.4. Lung Adenoma and Adenocarcinoma Incidence by Sex and Radiation Pretreatment

Treatment	Sex			
	Male		Female	
	Radiated	Nonradiated	Radiated	Nonradiated
Washed air chamber mice	7	3	4	1
Total number of tumors	10		5	
Smog chamber mice	10	15	4	1
Total number of tumors	25		5	
Chromic oxide chamber mice	2	8	6	0
Total number of tumors	10		6	

30.36 REQUIREMENT FOR CONTINUOUS ANTIGENIC STIMULATION IN THE DEVELOPMENT AND DIFFERENTIATION OF ANTIBODY-FORMING CELLS: THE EFFECT OF PASSIVE ANTIBODY ON THE PRIMARY AND SECONDARY IMMUNE RESPONSE

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Mary W. Francis

The levels of both antigen and specific immunoglobulin are considered important regulatory mechanisms which determine the size of antibody-producing cell compartments. Recent studies suggest that repeated antigen contact of the immunocompetent cells is required for complete differentiation. This concept of the continuous role of antigen in the immune response is consistent with the increasing body of evidence demonstrating that antigen does localize and persist in the lymphatic tissue during the primary immune response. One site in lymphatic tissue in which antigen has been demonstrated to be localized extracellularly in plasma membrane infoldings of unique reticular cells is the germinal center. Although the functional significance of this antigen deposition is still not completely resolved, experimental evidence demonstrates that there may be a causal relationship between the antigen localization and the presence and proliferation of characteristic large pyroninophilic cells of these centers. These cells, by morphologic and functional parameters, are considered immunologically competent cells. We have further suggested that during the primary immune reaction the persistence of antigen in germinal centers is

a dynamic process which is sensitive to and altered by the level of free specific antibody. In the present study we attempted to induce maximum immune progenitor cell conversion by using high antigen doses. Subsequently antigen was depleted at various intervals after priming with isologous specific antibody in order to interrupt further immune cell differentiation. It was reasoned that this condition would result in depression of the functional antibody-producing cell compartment, as measured in the intact animal, and subsequently an enhancement of the sensitized immune cell compartment, as assayed in the spleen cell transfer system. The latter is assumed to occur if an antigen is required for the continued differentiation of immunocompetent cells. These data were also correlated with a systematic study of the hyperplasia of spleen germinal centers in an attempt to better categorize the proliferating lymphoid cell compartment of these centers and to obtain further information concerning the role of the antigen localized in these centers.

Methods. – Male $BC3F_1$ mice were used in these studies. Animals were immunized with sheep erythrocyte antigen (SRBC). All injections were intraperitoneal (ip). Specific isologous antiserum was prepared by using the cell transfer method. The serum was diluted with saline to achieve 12 $\log_2$ titer units of hemagglutinin against the test antigen. The antiserum was found in a preliminary study to have a half-life of 12 days in $BC3F_1$ mice. The antiserum was also fractionated on a sucrose gradient, and essentially all activity was found in fractions previously established to contain protein less than 19S in size. Therefore the specific antibody is assumed to be primarily 7S globulin. Individual serum samples were titrated for hemagglutinin as well as hemolysin antibody. The hemolytic plaque-forming technique was used in this study. This study is a quantitative measure of cells producing hemolysin antibody.

Results and Discussion. – The effect of passive antibody on the primary response to SRBC as measured with the PFC assay was a marked decrease in the direct and indirect hemolysin-producing cells (DPFC and IPFC). However, there was a lack of correlation in the degree of antibody mediated DPFC (19S) and IPFC (7S) immune cell suppression during the primary response, the DPFC being much less depressed than the IPFC. As measured in the transfer system there was an enhanced 19S cell compartment and a depressed 7S cell compartment in one-day passively immunized mice. This was true regardless if transfers were performed at one, two, or four weeks after priming. Similarly there was an enhanced 19S cell compartment with little or no effect on the 7S sensitized cell compartment in four-day passively immunized mice. These data suggest that progeny of the antigen-stimulated progenitor cells, as a consequence of further lack of antigenic stimulation, were forced into maturation arrest.

These studies further demonstrate that isologous passive antibody suppresses germinal center growth regardless of whether the antibody is infused one, two, or four days after priming. In terms of formation of sensitized cells the marked depression of the 7S sensitized cell compartment after passive immunization in 24 hr, in contrast to the enhancement of the 19S sensitized cell compartment, corresponds to the suppressed growth of germinal centers during the primary response. Thus, if the germinal center is, as suggested, the site of proliferative expansion of immunocompetent cells, these data indicate that the center growth is related to the 7S antibody response and in the formation of "7S memory."

30.37 A COMPARATIVE STUDY OF THE IMMUNE REACTION IN GERMFREE AND CONVENTIONAL MICE

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H. E. Walburg, Jr.[1]

In recent years considerable attention has been devoted to the germinal centers of lymphatic tissue, particularly as these are structures related to the immune reaction. Results of studies performed by our group as well as others have demonstrated (1) that an antigen-stimulated proliferation of large pyroninophilic cells occurs in germinal centers and this proliferation is antigen dose dependent; (2) that the sensitized immune cell compartment develops first in lymphatic nodules at the time of germinal center hyperplasia; and (3) that during the course of an immune reaction, antigen is localized and retained extracellularly in plasma membrane infoldings of specialized reticular cells which constitute the fixed stroma of the germinal center.

A complicating factor in these immunologic studies of conventional animals is a chronic stimulation by adventitious antigens, resulting in formation of germinal centers in the spleen, Peyer's patches, and most lymph nodes. Ideally, detailed studies concerned with the growth, development, and functional significance of germinal centers require antigenically nonstimulated lymphatic tissue. The closest approximation to such ideal conditions is the germfree animal.

The purpose of the present investigation was to compare the immune capacity of intact germfree and conventional mice, in particular with regard to the

growth and development of lymphatic tissue germinal centers.

Methods. – Carworth Farm No. 1 (CF No. 1) mice were reared and maintained in the Biology Division of the Oak Ridge National Laboratory. Conventional mice used were CF No. 1, 10 to 12 weeks of age. The antigens used were sheep erythrocytes (SRBC) and ^{125}I-human gamma globulin (HGG).

At various intervals after antigen injection, animals were killed by cutting the throat, and blood was collected. One-tenth-milliliter serum aliquots and whole spleens of animals receiving ^{125}I-HGG were counted for radioactivity in a Packard Autogamma counter. Sections of spleen, liver, kidney, and mesenteric lymph node were used for autoradiography. Determinations of surface areas of the white pulp germinal centers were made by planimetry in each longitudinal spleen section. The in vivo culture system was used to follow the growth and development of the secondary antibody-forming potential.

Results. – *Primary Response to Sheep RBC.* – The latent and log phase of the hemagglutinin response was not significantly different in germfree and conventional mice. From day 6 on, the antibody level in germfree mice was consistently one to two $\log_2$ units above that in the conventional animals. Once peak titer was achieved in both groups, the hemagglutinin levels were relatively stable for the following three months.

The mean germinal center surface area increased approximately 2×10^3 μ^2 in conventional animals during the first ten days after injection of SRBC. During the same interval, the change in the germfree animals was approximately 4×10^3 μ^2. Thus there was a difference of twofold in the overall growth of germinal centers in germfree, as compared with conventional, mice. The peak size of the germinal centers in germfree animals at day 10, however, was not significantly different from that of the conventional animals at the same interval. By day 20 the mean germinal center surface area in both groups decreased to 2×10^3 μ^2, which is approximately equal to the background level in conventional mice. However, the germinal centers of germfree animals continued to decrease during the following three months.

Secondary Antibody-Forming Capacity in Germfree Mice. – One week after injection of SRBC, the spleen cells of germfree donor mice gave a six-day hemagglutinin response which was significantly above that produced by an equivalent number of spleen cells from unprimed donors, indicating that secondary antibody-forming potential was developing. Maximum level of the secondary antibody-forming capacity, however, was not reached before 30 to 60 days after priming. This peak level was maintained until five months after antigenic stimulation, at which time the experiment was terminated.

Histology and Autoradiography During the Primary Response to HGG. – One hour after injection of 1.5 mg ^{125}I-HGG in conventional mice, label was found preferentially in the marginal zones of spleen lymphatic nodules. At 6, 12, and 24 hr, the preexisting germinal centers were heavily labeled, while label decreased in other areas of the spleen (Fig. 30.37.1*A* and *B*). Between 2 and 20 days after antigen injection, the label was predominantly concentrated in germinal centers though the concentration decreased progressively with time. The overall pattern of antigen localization during the first 10 days was similar in the germfree mice as compared with the conventional mice. The major difference, however, was that although label concentrated in defined perifollicular areas of the lymphatic nodules, no active germinal centers existed (Fig. 30.37.1*C* and *D*). However, between 10 and 20 days, germinal centers formed in these regions of antigen retention. In the present study there was no deficiency in hemagglutinin-forming potential, development and growth of lymphatic tissue germinal centers, or antigen localization as well as antigen clearance in germfree mice compared with conventionally reared animals of the same strain. Rather, the data of these experiments suggest that the germfree mice may have an increased immune capacity. However, since the level of antigenic background stimulation in the conventional mouse is not controlled, the magnitude of this difference, if it is real, could be expected to vary among experiments.

Conclusion. – Based on the sequence of histologic and serologic events observed in this serial study of the primary response in germfree mice, we would suggest the following: Lymphatic tissue has two unique components, dispersed immunologically competent progenitor cells and defined areas of reticular cells, capable of localizing antigen extracellularly. After the initial antigenic stimulation, one phase of the proliferative expansion of the immunologically competent progenitor cells occurs in the region of the antigen-retaining reticular cells. This results in the accumulation of large pyroninophilic cells in this area and ultimately in the formation of a histologically well-defined germinal center. At the same time antibody becomes detectable in the serum. The serum antibody response and the growth of the germinal center simultaneously develop to peak levels. During this time the sensitized cell compartment is being established in the lymphatic nodules. Therefore the proliferation of large pyro-

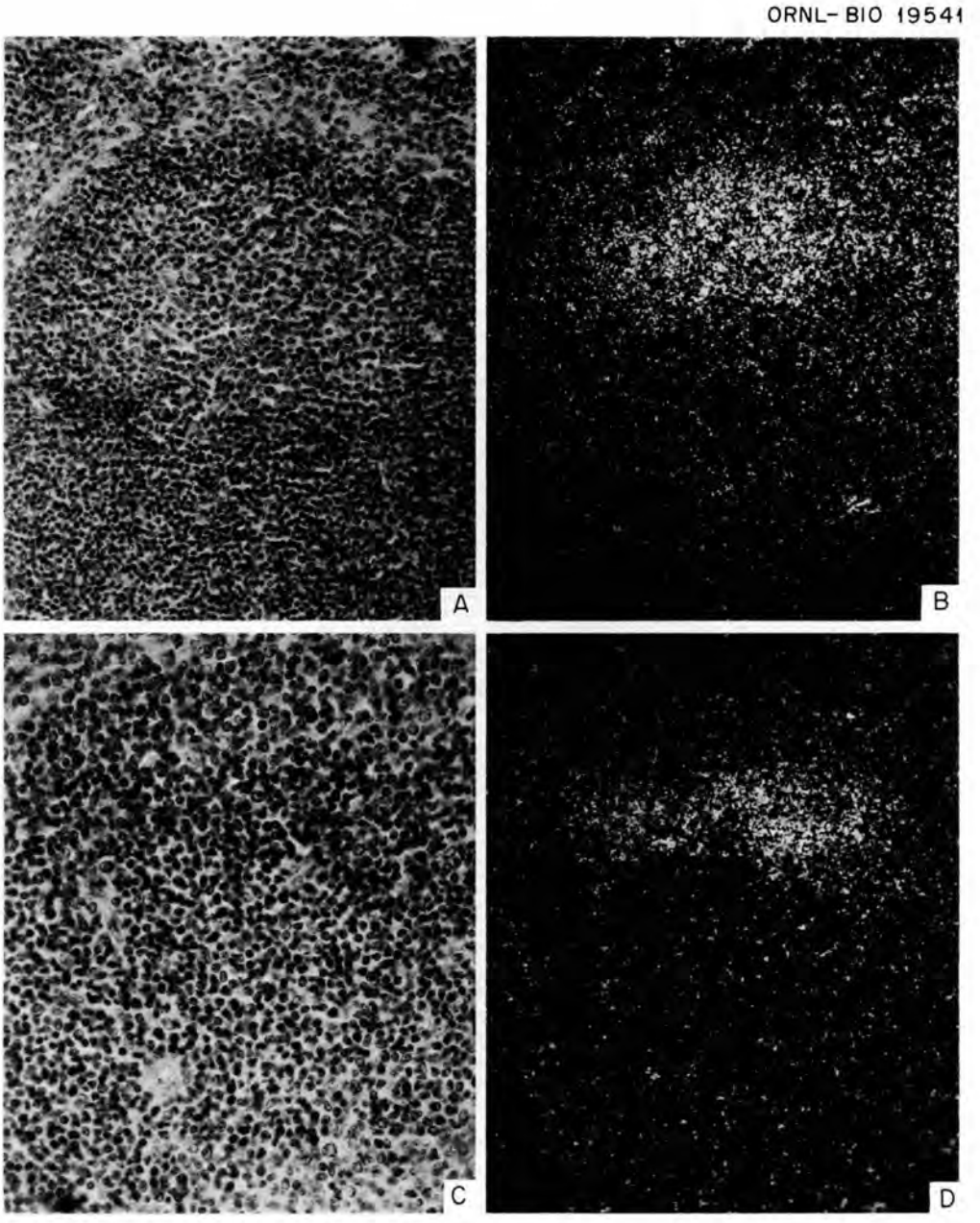

Fig. 30.37.1. ^{125}I-HGG Localization in Spleen Lymphatic Nodules During the Primary Response. *A*, active germinal center in spleen of conventional mouse 12 hr after injection; *B*, dark field photomicrograph of an autoradiogram of the identical area in the adjacent section; *C*, perifollicular region of lymphatic nodule in germfree spleen 12 hr after injection; *D*, dark-field photomicrograph of an autoradiogram of the identical area in the adjacent section.

ninophilic cells in germinal centers is probably related to both the development of antibody-producing cells as well as memory cells. Since the largest proportions of antibody-producing cells and memory cells are eventually found outside the germinal centers, the lymphoid cell population of the centers must be considered transitory. This is also supported by our previous kinetic studies of active germinal centers. The later decrease in the size of the centers probably is a manifestation of the diminishing growth rate of the antibody response as well as the memory formation. This may be a consequence of the elimination of antigen from these areas.

Reference

[1] Pathology and Physiology section.

30.38 LOCALIZATION OF ^{125}I-LABELED ANTIGEN IN GERMINAL CENTERS OF MOUSE SPLEEN: HISTOLOGIC AND ULTRASTRUCTURAL AUTORADIOGRAPHIC STUDIES OF THE SECONDARY IMMUNE REACTION

M. G. Hanna, Jr. A. K. Szakal

It has been demonstrated that during the immune reaction, antigen localizes extracellularly in plasma membrane infoldings of dendritic reticular cells located in the lymphatic tissue germinal centers. We have further demonstrated that during the primary immune reaction the persistence of antigen in germinal centers is a dynamic process which is sensitive to, and altered by, the level of specific antibody. Thus an exaggerated but synchronous response would occur when antigen is given in the presence of specific serum antibody in actively immunized animals where, although there is initial localization of antigen, the persistence in germinal centers is relatively short compared with the primary immune response.

The levels of both antigen and specific antibody are considered regulating mechanisms with regard to magnitude of the antibody-producing cell compartment. One mechanism of the selective activity of antibody would be through competition with immunologically competent cells for antigen. This selective activity would then be maximum during the secondary response and, to be optimally effective, would have to minimize or block developing and/or developed immunologically functional antigen-trapping mechanisms. Accordingly, the purpose of this investigation was to systematically study, on a histologic and ultrastructural level, ^{125}I-labeled human gamma globulin (HGG) localization in mouse spleen germinal centers during the secondary immune reaction in animals having a preexisting hemagglutinin titer.

Materials and Methods. – Male BC3F$_1$ [(C57BL ♀) X (C3H/AN ♂)F$_1$ Cum] specific-pathogen-free (SPF) mice 12 to 14 weeks of age were used. Groups of mice were primed at 0 time and at three days with 1.5 mg HGG. Animals used for the serologic and histologic studies were divided into two groups, with one group receiving 1.5 mg ^{125}I-labeled HGG on day 44. Five mice were killed at 2 hr, 12 hr, and days 1, 2, 3, 4, 5, 8, and 12 after the second injection of antigen. Twenty primed animals were not injected for a secondary response, and these were killed in groups of five at days 30, 45, 52, and 62 after priming.

One-tenth-milliliter samples of serum were used for counting radioactivity, and the remainder of the serum was frozen for passive agglutinin titrations. Tissue (such as spleen, measured pieces of liver, and kidney) to be assayed for radioactivity was placed in tubes containing 1 ml Bouin's fluid. All counts were made in a Packard Autogamma counter. Tissue sections were prepared for autoradiography. Electron-microscopic autoradiography was performed on thin sections coated with Ilford L-4 nuclear research emulsion. The ultrathin sections were examined in the HUIB Hitachi electron microscope after development in Kodak Microdol X.

Histologic Autoradiography of the Secondary Response. – During the first 24 hr after the secondary injection of antigen the most dramatic histologic change was the accumulation of label in the spleen germinal centers. The amount of label in the germinal centers markedly decreased between days 2 and 3, and concentration of grains was seen in central arterioles of the lymphatic nodules. Also at this time spotty concentrations of grains were detected in the cortical lymphocyte zone of the nodules. No significant labeling could be seen in the periarterial lymphocyte mass of the nodule or in the spleen red pulp. The three-day interval corresponds to the peak retention of label counted in the spleens, and it was assumed that the majority of the grains seen at this time were associated with antigen-antibody complexes.

To better define the location of grains in the lymphatic nodules at three days after the secondary injection, studies were made of autoradiograms of 1-μ sections of Epon-embedded material. It could be seen in these autoradiograms that the grains were concentrated in capillaries of the cortical lymphocytic zone and in the central arterioles of the nodules. At days 8 to 15 the only concentrations of grains in the spleen were

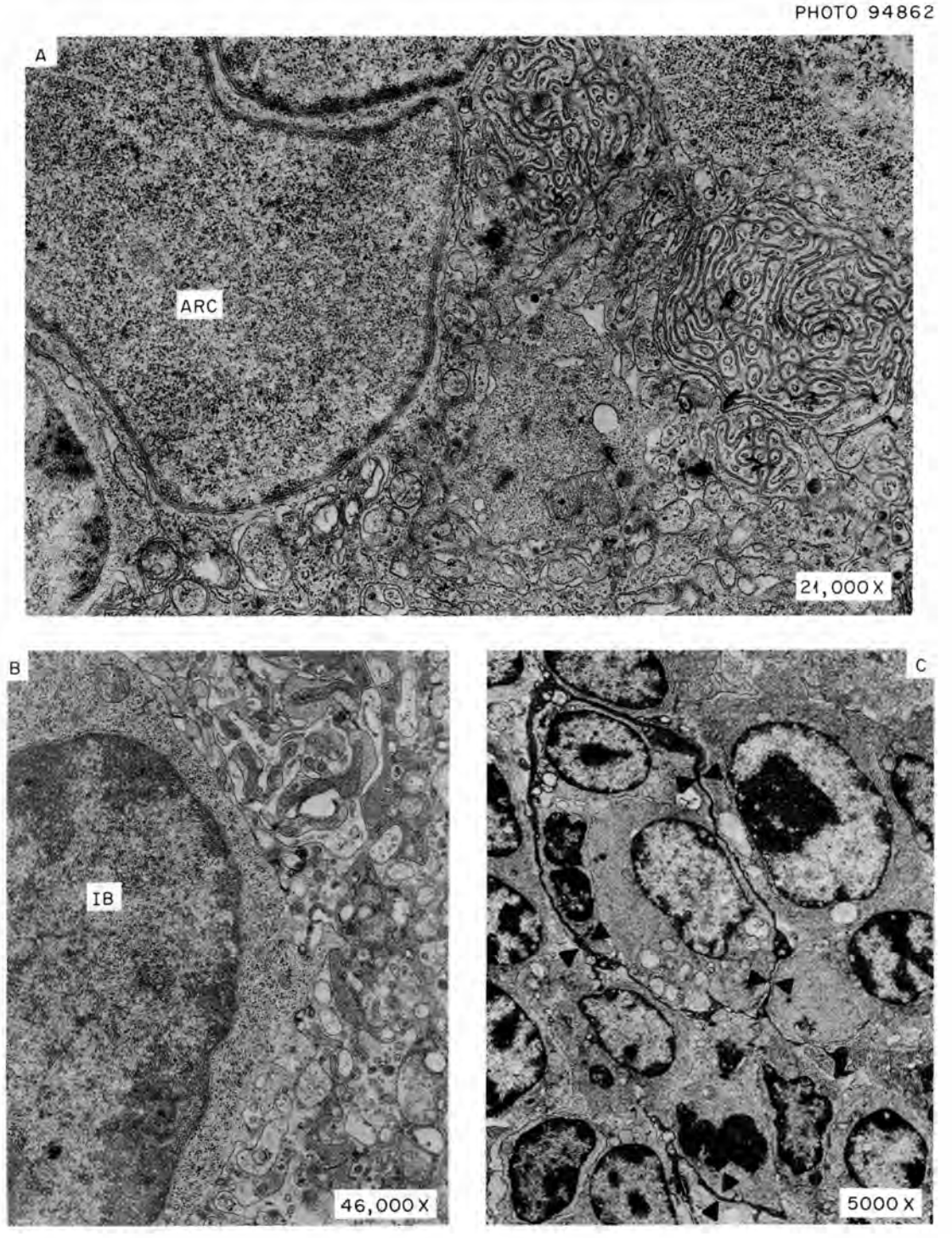

Fig. 30.38.1. *A*, **Microautoradiograms Illustrating the Distribution of Silver Grains over the Labyrinthine Plasma Membrane Infoldings of Antigen-Retaining Reticular Cell Processes (ARC) 2 hr After the Initiation of the Secondary Immune Response in Mouse Spleen Germinal Centers.** Note the uniformity of the plasma membrane infoldings. 21,000X. *B*, an electromicrograph of a portion of a germinal center at day 3, demonstrating the dilation of the membrane infoldings of the antigen-retaining cell. Note the disruption in the continuity of the plasma membrane adjacent to an immunoblast. 46,000X. *C*, electron micrographs of highly electron-dense cytoplasmic remnants of antigen-retaining reticular cells seven days after secondary injection. (▲), remnants condensed among parenchymal cells of germinal centers. 5000X. Reduced 23%.

associated with the central arterioles. The germinal centers and lymphocyte mass of the nodules were essentially cleared of detectable grains.

Ultrastructural Studies. – At 2 hr after the secondary response injection of HGG, silver grains observed in germinal centers were primarily located overy very numerous plasma membrane infoldings of antigen-retaining reticular cells (Fig. 30.38.1*A*). The extensive cytoplasmic processes containing complex and labyrinthine infoldings of plasma membranes filled the intercellular space between the large parenchymal cells (immunoblasts) of the germinal center.

By day 1 after the secondary injection of antigen there was a loss of regularity and organization of the plasma membrane infolding (Fig. 30.38.1*B*). At day 3 the disorganization of the membrane infoldings and the formation of large pools of the electron-dense material were even more pronounced. By day 7 after injection of antigen some membranous electron-dense remnants of the antigen-retaining cells were seen forming continuous networks among the free parenchymal cells of the germinal centers examined. These remnants of the processes of antigen-retaining cells appeared to be condensed among the free cells (Fig. 30.38.1*C*).

Conclusion. – The magnitude and regularity of the developed plasma membrane infoldings of antigen-retaining reticular cells in germinal centers are described. The key finding in this study was the degradation of these specifically developed plasma membrane infoldings during the early intervals of the secondary response. This response occurred in conjunction with a loss of label and an apparent loss of function of the existing antigen-trapping mechanisms in spleen germinal centers. We suggest that the cytotoxic sequelae described could be a result of complement fixation and that such a regulatory mechanism would be essential in preventing further contact of immunologically competent cells with antigen while clearing the centers for development against further antigens. A conformity of vesicles was observed in the cytoplasm of the parenchymal cells of the germinal centers and in the plasma membrane infoldings of the antigen-retaining reticular cells.

30.39 VIRUS PARTICLES IN SPLEEN GERMINAL CENTERS

M. G. Hanna, Jr. A. K. Szakal

A further aspect of the electron microscopic studies of spleen germinal centers during the immune reaction was the observation of large numbers of endogenous virus particles in the plasma membrane infoldings of antigen-retaining reticular cells. The particles measured 100 to 120 mμ in diameter, and many resembled the C-type virus particle associated with murine leukemia. Occasionally, budding of the C-type virus particles from the plasma membranes of the characteristic lymphoid cells was observed. During studies using ^{125}I-HGG, the numerous virus particles could be seen together with silver grains extracellularly in plasma membrane infoldings. This finding suggested a similarity in the mechanism of retention of the exogenous antigen and the apparently endogenous virus particles.

In light of the immunosuppressive effect of some murine leukemia viruses and their normal occurrence in spleen germinal centers, a systematic study of virus localization in germinal centers was performed. Young adult BALB/c mice received intraperitoneal injections of Rauscher virus preparation. The spleen, thymus, mesenteric lymph node, and bone marrow of these mice were studied histologically and electron microscopically at intervals between 6 hr and 20 days after injection. Numerous virus particles were observed localized extracellularly in plasma membrane infoldings of dendritic reticular cells of spleen and mesenteric lymph node germinal centers. The majority of these particles were of C type. The number of viruses in these germinal centers markedly increased between days 1 and 20 after injection. Budding of C-type particles from the parenchymal lymphoid cells of the germinal centers occurred with increasing frequency between days 1 and 20 after injection. Viruses were also observed in the thymus, bone marrow, and spleen red pulp of these experimental mice; however, the concentration of virus, even at 20 days after injection, was markedly less in these tissues compared with the concentration in germinal centers. This was also true when comparison was made between germinal centers and the associated lymphocyte mass of the lymphatic nodules.

Conclusion. – With the present state of knowledge it is impossible to determine the functional significance of virus localization in lymphatic tissue germinal centers. It is pertinent, however, that the observation was made for both endogenous as well as adventitious viruses. Also, as would be expected, the mechanism of extracellular localization (in plasma membrane infoldings of dendritic reticular cells) is identical to that which can be shown to develop for a variety of soluble and particulate antigens. A most interesting aspect of the virus localization in germinal centers is the prevalence of C-type particles and their proliferation in the lymphoid cells. In the situation of the Rauscher virus preparation in BALB/c mice, the selective retention of C-type particles might have initially been stimulated by an immune response, but ultimately resulting in the initial proliferative phase of the virus. In some way this finding must correlate to the immunosuppressive effect

of most murine leukemia viruses. Thus if the importance of antigen localization in germinal centers is to provide a unique opportunity for surface contact of antigen and lymphoid cells, then the present observations may be relevant to (1) immunologic recognition and response to viruses and (2) the opportunity of some viruses to find suitable target cells for proliferation. The latter is most relevant for murine leukemia viruses.

30.40 RADIOSENSITIVITY OF THE ANTIGEN TRAPPING MECHANISM AND ITS RELATION TO THE SUPPRESSION OF THE IMMUNE RESPONSE

Paul Nettesheim M. G. Hanna, Jr.

Our studies concerning regenerative potential after x irradiation of the cell compartment responsible for immunological memory indicated that recovery of this progenitor cell pool is dependent on the availability of uncommitted immune progenitor cells *and* persistence of antigen.[1] We therefore decided to study the effects of irradiation on the antigen-trapping and -retaining mechanism in greater detail during various stages of the immune response. The antigen chosen for this investigation was heat-aggregated ^{125}I-labeled human gamma globulin (^{125}I-HGG). We found that x ray given prior to antigenic stimulation did not completely abolish the antigen trapping in germinal centers; however, it caused an enhanced elimination of antigen from these areas. This x-ray-induced defect of the antigen retention was found to persist for several weeks (Fig. 30.40.1). The enhanced elimination of antigen could have been due to x-ray damage, either to cells responsible for trapping

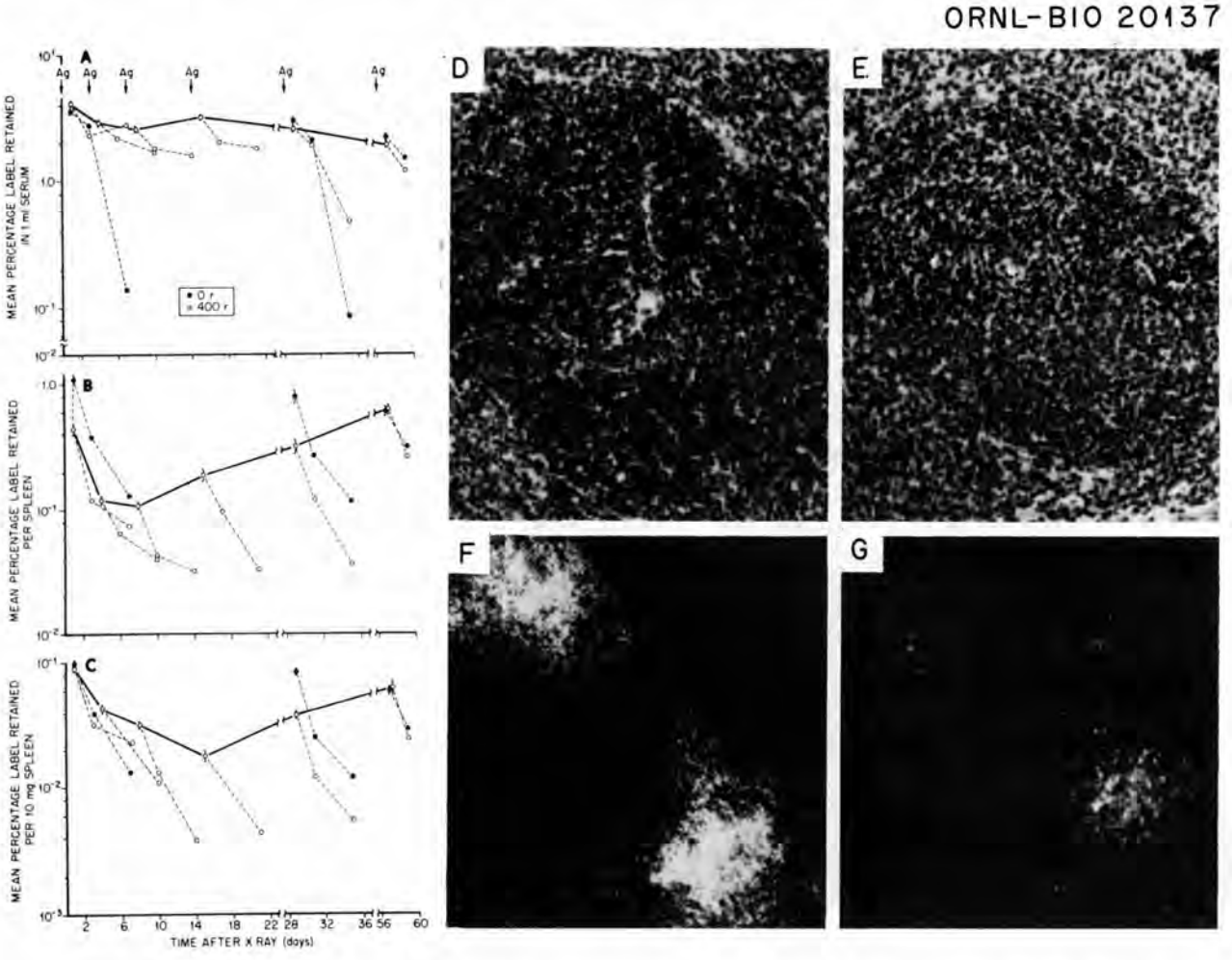

Fig. 30.40.1. Effects of X Irradiation on Retention of ^{125}I-HGG. (*A*) In serum; (*B*) in whole spleen; (*C*) per unit spleen weight: ○, irradiated mice; ●, unirradiated mice. (*D*) Spleen lymphatic nodule of unirradiated mouse; (*E*) four weeks after 600 r (H&E ~ 500X). Dark-field photomicrograph of spleen autoradiogram (~125X) one day after ^{125}I-AHGG, (*F*) of unirradiated mouse and (*G*) of mouse irradiated with 600 r four weeks previously. Enlarged 34%.

and retaining the antigen or to the immune progenitor cells, or both. In the latter case the enhanced elimination could have been a consequence of the delay of antibody production, since antibody is being implicated as a factor involved in antigen localization. To decide between these alternate possibilities, a second series of experiments was carried out in which x irradiation was given to animals with circulating 19S or 7S antibody specific for the test antigen. X ray did not affect the level of circulating antibody. It did, however, cause an enhanced elimination of the spleen in spite of the presence of specific antibody. Electron microscopic evidence suggestive of cellular damage of reticular cells implicated in antigen retention was obtained. Since these cells are known to have a low proliferative rate, this might explain the long-lasting defect in antigen-retaining capacity.

Reference

[1] P. Nettesheim and M. L. Williams, *J. Immunol.* **100**, 760 (1968).

30.41 REGENERATIVE POTENTIAL OF IMMUNOCOMPETENT CELLS INVOLVED IN THE PRIMARY AND SECONDARY ANTIBODY RESPONSE

Paul Nettesheim Mary L. Williams

The present study is a continuation of our previous attempts to characterize the various immune progenitor cell compartments stimulated in the primary and secondary antibody response. At least two distinct antigen-sensitive precursor cells are postulated in the current cytokinetic models of the antibody response. The criterion by which we hoped to distinguish between the different progenitor cell pools, if they indeed exist as separate entities, was their regenerative capacity as established during the recovery phase from x irradiation. The in vivo culture system of lymphoid cells was applied in this study, since it allows an estimation of the immune progenitor cell pool size. Figure 30.41.1 summarizes the main findings of these studies, represented by three major patterns of recovery. The primary antibody-forming potential (curve *A*) recovers very slowly from x irradiation, eventually reaching a normal level. This process does not require antigen and is, as will be discussed in more detail later, partially thymus dependent. In contrast, previous investigations revealed that the secondary antibody-forming potential does not recover when antigen is either no longer available or at too-low concentrations

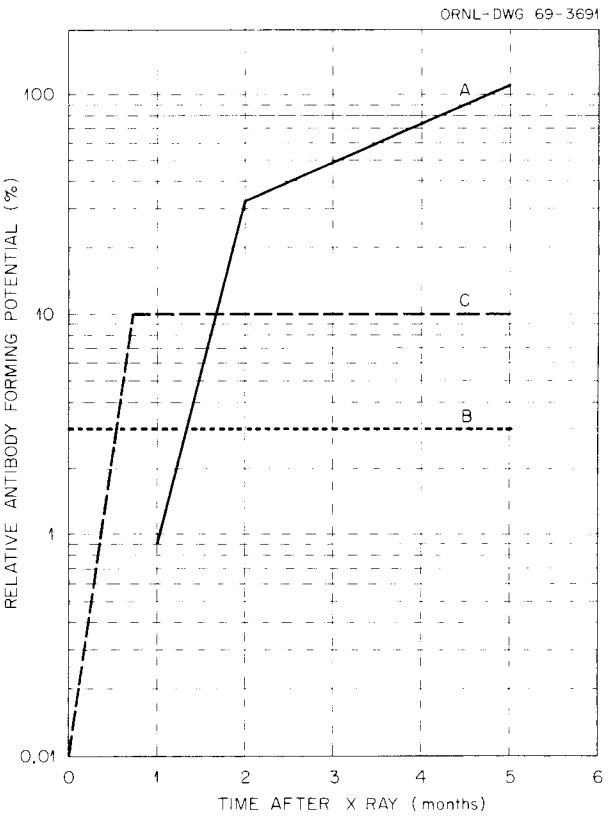

Fig. 30.41.1. Schematic Representation of the Three Major Recovery Patterns of the Immune Capacity, Observed After Sublethal X Irradiation. Primary antibody forming capacity (——). Secondary antibody forming capacity, short priming, irradiation interval (– – –). Secondary antibody forming capacity, long priming, x-irradiation interval (...).

(curve *B*), though a fast but only partial recovery is observed when antigen continues to be present for some time after the x-ray insult (curve *C*). This indicates that the cell pool which is initially triggered by antigen in the *primary* response is distinct from the cell pool predominantly responding in the secondary response. The former is part of a self-renewal system, normally maintained in a steady state, which recovers after x irradiation; the latter is a specific differentiation product of the former induced by antigenic stimulation and does not recover from injury if this stimulus is lacking. As the antigenic stimulus subsides, the production ceases. Since its self-generating capacity appears to be limited, it can be permanently reduced in size or even be abolished by insults such as x irradiation.

We then studied the effect of the thymus on the recovery of the primary immune potential in x-irradiated mice. Young adult mice were thymectomized and

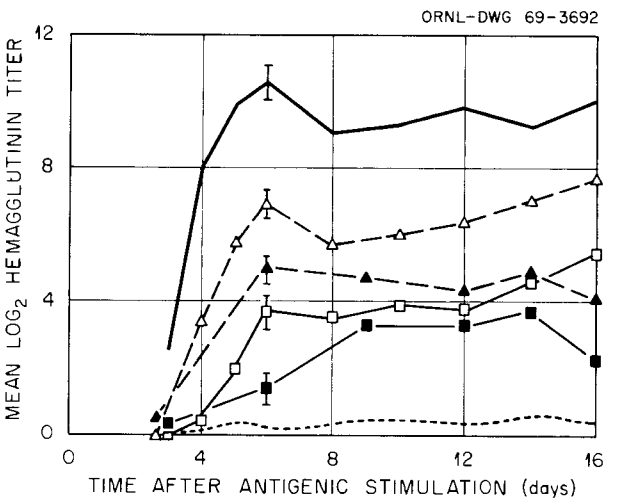

Fig. 30.41.2. Recovery of the Primary Rat Hemagglutinin Response of Normal and Thymectomized $C3_1F_1$ Mice from 600 r Whole-Body Exposure. 10^9 rat RBC were injected ip into unirradiated mice (——), and irradiated mice at the following intervals after x irradiation: 2 hr (.....); 4 weeks, normal (□–□), thymectomized (■–■); 8 weeks, normal (△–△), thymectomized (▲–▲). Microtitration method; 10 to 12 samples per point.

irradiated with sublethal x-ray exposures two weeks later. At various times thereafter an antibody response was induced by injecting 10^9 rat red blood cells. The results summarized in Fig. 30.41.2 show that recovery of immune competence is slower in thymectomized irradiated mice than in nonthymectomized irradiated controls. However, recovery from the x-ray-induced immune suppression is by no means abolished in thymectomized animals. This indicates that the immunologically competent cell or unit (since probably more than one cell type is required for initiation of the antibody response) can regenerate in the absence of the thymus, though the rate of regeneration is thymus dependent.

30.42 STUDIES ON THE MAINTENANCE OF THE LATE ANTIBODY RESPONSE

Paul Nettesheim Anna S. Hammons

Immunological memory and high levels of serum antibody are maintained for many months in animals immunized with various types of antigen. One proposed mechanism for this lengthy immunity is that antigen persists for many months in the lymphoid organs and in other organs, and continuously triggers immune progenitor cells to form new antibody-producing cells and/or memory cells to replace dying ones. Such a renewal system is particularly useful in explaining the continued antibody response, since the half-life of 7S hemagglutinating antibodies in the serum of mice has been found to be approximately four days, and that of most antibody-producing cells two to five days. X ray has been shown to interfere with antigen-induced cell proliferation and differentiation, but not with antibody production itself when given at peak of response. It was therefore used to test whether the high serum antibody levels found late in the immune response are dependent on continuous recruitment of antibody-forming cells. Mice thymectomized during adulthood were immunized with heterologous red blood cells and were irradiated four to eight weeks after immunization (i.e., at plateau of antibody response) with either 2 X 400 r (three weeks apart) or 1 X 850 r (+ 2 X 10^6 isologous bone marrow). Thymectomy was performed prior to x irradiation since it has been shown to prolong the x-ray-induced immune suppression. It was found that within the five to eight weeks following the x-ray exposure the decay rate of circulating antibody was only slightly faster in irradiated than in unirradiated control animals. In other experiments Millipore diffusion chamber cultures of spleen cells undergoing a secondary antibody response were irradiated in vitro with 1200 r on plateau of antibody response. The chamber cultures were implanted into mice and transferred every two weeks to a new set of recipients. The level of circulating antibody was measured in these recipients. It should be noted that with each chamber transfer into a new host the chamber culture (volume 0.15 ml) has to release enough antibody in order to

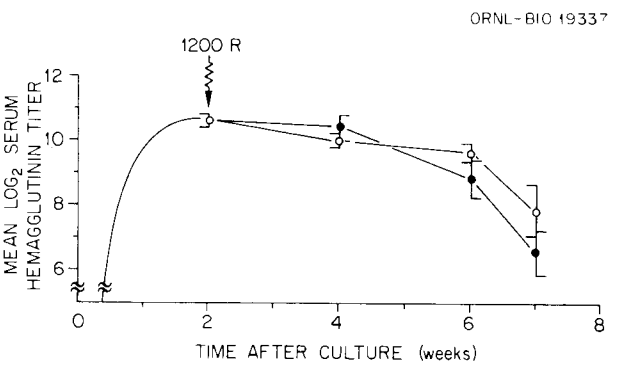

Fig. 30.42.1. Effect of X Irradiation on Serum Hemagglutinin Levels of Mice Carrying Irradiated (●) or Unirradiated (○) Diffusion Chamber Cultures. Cultures were inoculated with 25 X 10^6 primed spleen cells and 2.5 X 10^6 sheep red blood cells and irradiated with 1200 r two weeks later (see arrow). There were five to eight samples used per point. Horizontal bars indicate standard error of the mean.

raise the recipients' serum antibody from zero to levels between six and ten $\log_2$ titer units (total body fluid per 25-g mouse ~2 ml). It seems reasonable to assume that this can be accomplished only if synthesis of antibody is taking place.

The results of this experiment are summarized in Fig. 30.42.1. It can be seen that no significant differences in antibody levels were detectable between mice carrying either unirradiated or irradiated cultures. The results strongly suggest that the maintenance of the serum antibody plateau late in the immune response is dependent on a long-living population of antibody producing cells and that recruitment of such cells, if occurring at all during this phase of the response, must be very slow.

30.43 THE FATE OF 1-(4-DIMETHYLAMINOBENZYLIDENE)INDENE IN RATS

C. T. Bahner D. G. Doherty

Study of the fate of 1-(4-dimethylaminobenzylidene)indene (I) in vivo has been continued. I labeled with tritium on the aromatic ring of the benzylidene portion of the molecule was dissolved in peanut oil and administered ip to normal and tumor-bearing female rats. After two to three days the animals were killed, and tissues were taken for preparation of autoradiograms. Formalin-fixed bone marrow smears showed a high level of radioactivity. Frozen tissues were heavily radioactive, but diffuse blackening of the slides, and streaks, indicated that the radioactivity was leaking from the specimens in soluble or colloidal form. Formalin-fixed tissues embedded in paraffin and sectioned to make slides retained much less radioactivity, but it was more clearly localized. Fat cells in the abdomen were heavily labeled. Red cells in the blood vessels were consistently labeled. Liver and spleen were well but diffusely labeled. Normal liver cells retained more radioactivity than did leukemic Lymphoma 8 cells, which invaded the liver. Walker 256 tumor cells seemed more radioactive than Lymphoma 8 cells in the solid tumors, but both were well labeled. Uterus, ovary, and vagina were labeled especially in the follicular area and epithelial lining. Islet and acinar cells of the pancreas and the cortex and medulla of adrenals and thymus were radioactive. Lung and kidney tissues were moderately active. Hair follicles were consistently more radioactive than other nearby skin. In some instances there was selective labeling of one layer of the retina. Layers of cells in artery walls were labeled, but the elastic membrane in the walls was almost free from radioactivity.

30.44 TRANSLATIONAL AND TRANSCRIPTIONAL CONTROL OF TYROSINE-α-KETOGLUTARATE TRANSAMINASE SYNTHESIS IN CULTURED HEPATOMA CELLS

Kai-Lin Lee[1] F. T. Kenney

The level of tyrosine transaminase in cultured Reuber H-35 hepatoma cells is inducible by at least two and probably three discrete induction mechanisms, mediated by hydrocortisone, glucagon, and insulin respectively. Previous studies in this laboratory[2] have suggested that the transaminase mRNA in these cells may be sufficiently stable to permit discrimination between transcriptional and translational mechanisms of induction. The present experiments have revealed the following:

1. The early phases of induction by glucagon are insensitive to actinomycin D, while induction by hydrocortisone is always sensitive to this antibiotic.
2. When cells are preinduced by hydrocortisone a limitation in the response to glucagon is removed, and glucagon effects a very rapid and large induction which is completely insensitive to actinomycin.
3. Preinduction of cells with glucagon does not alter the response to subsequent addition of hydrocortisone.
4. Brief exposure of cells to hydrocortisone and subsequent removal of the hormone causes the transaminase to increase continuously for another 2 hr. Under identical experimental conditions but with pretreatment with glucagon rather than hydrocortisone, the transaminase activity immediately dropped when the glucagon was removed.
5. When hydrocortisone and glucagon were added together, the increase in transaminase was more than the additive response to each hormone alone. These results clearly indicate that the steroid hormone induces tyrosine transaminase by promoting the synthesis of transaminase mRNA, while glucagon acts at some post-transcriptional step in enzyme synthesis.

References

[1] Biochemical Regulation section.

[2] J. R. Reel and F. T. Kenney, *Proc. Natl. Acad. Sci. U.S.* **61**, 200 (1968).

30.45 HORMONAL REGULATION OF TYROSINE TRANSAMINASE IN FETAL RAT LIVER IN ORGAN CULTURE

W. D. Wicks

It now appears likely that there are three discrete mechanisms for inducing hepatic tyrosine transaminase, involving hydrocortisone, insulin, and cyclic AMP as primary inducers. Thus, when combinations of these inducers are added to explants of fetal liver in organ culture, the response of tyrosine transaminase activity is as follows: synergistic with hydrocortisone and cyclic AMP, additive with hydrocortisone and insulin, and somewhat less than additive with insulin and cyclic AMP. The synergistic response to the combination of steroid and cyclic AMP is visualized as reflecting a sequential stimulation of enzyme synthesis by the two inducers. Kinetic studies and evidence from other systems support such a possibility, and the data suggest that the steroid enhances transaminase messenger RNA formation, while cyclic AMP promotes the decoding of this messenger in some manner. The site at which insulin mediates the synthesis of the transaminase is presently unknown, but it seems clear from the combination studies and other data that it must be distinct from those affected by hydrocortisone and cyclic AMP. The lack of complete additivity in the response to insulin and cyclic AMP can be accounted for by the well-documented antagonistic effect of insulin on cyclic AMP in liver.[1] If insulin induces the transaminase by one means and independently acts to block the induction by cyclic AMP, the results would be readily explained. At present it is not clear why both insulin and cyclic AMP should induce the same enzyme, when they normally act in opposition. Epinephrine biosynthesis ultimately depends upon the supply of blood tyrosine, and it is conceivable that induction of the primary enzyme involved in tyrosine breakdown could act to reduce the availability of tyrosine and consequently lower the rate of synthesis of epinephrine. In the case of insulin this would indirectly suppress the gluconeogenic action of epinephrine on the liver. In the case of cyclic AMP, induction of the transaminase would act as feedback control since epinephrine stimulates the synthesis of this nucleotide in liver.

Experiments are now in progress aimed at delineating, at the molecular level, the different mechanisms of induction of this enzyme.

Reference

[1] L. S. Jefferson *et al., J. Biol. Chem.* **243**, 1031 (1968).

30.46 ENZYMES AND FACTORS REGULATING THEIR SYNTHESIS IN HEPATOMA CELL CULTURES[1]

Kai-Lin Lee J. R. Reel F. T. Kenney

Introduction. – Most of the work on regulation of enzyme levels in normal tissues or in tumors has been done in vivo, with consequent uncertainty as to the actual factors responsible for regulating the levels of the enzymes examined. We have begun to analyze the various inducible liver enzymes in cells of the H35 hepatoma grown in tissue culture. Such an approach is essential to an understanding of hormonal regulation of enzyme synthesis, particularly in identifying with certainty which enzymes or groups of enzymes are sensitive to particular hormones. Also, it may be possible by this means to identify changes in the cultured cells which are of significance in neoplasia.

Results. – *Tryptophan Pyrrolase.* – This enzyme is inducible by glucocorticoids in vivo, in liver perfusion studies, and in the H35 hepatoma grown in vivo. In our cultured cells pyrrolase activity is not detectable, whether the cells are steroid-treated or not.

Serine Dehydrase. – This enzyme has been reported to be induced in vivo and in hepatomas grown in vivo by a variety of agents (glucocorticoids, glucagon, epinephrine, amino acid mixtures, etc.), of which we regard glucagon (cyclic AMP) as the effective agent. In our cultured cells no serine dehydrase activity could be detected, even after treatment with glucagon or hydrocortisone.

Alanine Transaminase. – This enzyme is induced in livers of glucocorticoid-treated rats, but no data have been reported to indicate that the steroid effect is direct. The enzyme level in our cultured cells is approximately the same as in the liver in vivo. Supplementation with glucocorticoids causes a two- to threefold increase in this level, but several factors suggest that this may not reflect increased synthesis of the enzyme. This point will be examined using an antiserum prepared against alanine transaminase and provided to us by Dr. H. L. Segal of SUNY, Buffalo, New York.

Glucose-6-phosphatase. – This enzyme has been reported to be induced in vivo by glucocorticoids and by glucagon and other factors which we regard as secondary to glucagon release. The phosphatase activity in our cultured cells is very low and could not be increased either by hydrocortisone or by glucagon.

Glucokinase. – This enzyme has been reported to be induced in vivo both by insulin and by glucose; as these factors are closely interrelated in vivo, there has been much controversy over the question of which is the

actual effective agent. In our cultured cells we have been unable to obtain an adequate assay for this enzyme. The assay employed involves coupling to glucose-6-phosphate dehydrogenase and measurement of TPN reduction; in the cultured cells a very active "nothing dehydrogenase" rapidly reduces TPN and obscures the glucokinase reaction.

Discussion. – From these preliminary results it is apparent that no consistent pattern has yet been established. The tyrosine transaminase in cultured hepatoma cells is present in normal amounts and responds to all of the inducing hormones previously studied in vivo, namely, hydrocortisone, glucagon, and insulin. But hydrocortisone will not induce tryptophan pyrrolase and has at best a small effect on alanine transaminase. Glucagon will induce neither serine dehydrase nor glucose-6-phosphatase. From these results the important implication is emerging that the hormones may be unable to activate genes which are thoroughly inactivated; rather, they can alter rates of transcription or of translation but only of active genetic sites. This is consistent with other aspects of hormonal regulation of enzyme synthesis.

Reference

[1] For appropriate literature citations see "Hormonal Regulation of Synthesis of Liver Enzymes," a chapter in *Mammalian Protein Metabolism,* vol. 3, ed. by H. N. Munro, Academic, New York, in press.

30.47 "SUPERINDUCTION" BY ACTINOMYCIN D: THE RESULT OF DIFFERENTIAL INHIBITION OF ENZYME SYNTHESIS AND ENZYME DEGRADATION

J. R. Reel F. T. Kenney

The tyrosine transaminase of cell cultures of minimal deviation hepatoma cells is induced by hydrocortisone. This effect is blocked if actinomycin is added early in the course of induction, which is consistent with the concept that the steroid hormone promotes transcription. If actinomycin is withheld until the hormonal induction has reached the new steady state typical of elevated enzyme synthesis, the antibiotic causes a marked further increase in the enzyme level, an effect which has been termed "superinduction." This has now been reported in a variety of mammalian regulatory systems, and it has been attributed to the action of cytoplasmic repressors acting to limit the extent of hormonal induction.

We have measured rates of synthesis and degradation of tyrosine transaminase in both H35 and HTC cells subjected to the "superinduction" treatment. In both cell lines transaminase synthesis was inhibited by the antibiotic, falling off exponentially with an approximate half-life of 3 hr. Despite the inhibition of synthesis the enzyme level was increased. This disparity reflects a marked and rapid inhibition of degradation of the enzyme, which was quickly reduced to about 25% of the normal rate. As there is a period of 2 to 3 hr during which synthesis of the enzyme is high (though steadily dropping) and degradation is slowed, the enzyme level rises. The data of these experiments fit remarkably well the theoretical formulation

$$dE/dt = k_1 - k_2 E ,$$

where k_1 is the rate constant of synthesis and k_2 is the rate constant of degradation. This has hitherto been thought to be a rather crude approximation.

These data provide a further indication that the process of enzyme degradation is complex, as we have shown in previous in vivo experimentation. They also show clearly that "superinduction" in this experimental system is not indicative of translational repression.

30.48 HORMONAL REGULATION OF ENZYME SYNTHESIS IN CARCINOGEN-TREATED RATS

F. T. Kenney L. E. Roberson
Joseph Kendrick G. R. Holloway

A group of 100 pathogen-free rats were fed the hepatic carcinogen diethylnitrosamine under conditions in which the chronology of pathological changes has been documented. At intervals, groups of rats were adrenalectomized and then tested for the capacity of the liver to respond to two different types of hormonal inducers of enzyme synthesis. These were (1) the steroid hydrocortisone, which we believe acts by promoting transcription, and (2) the polypeptide glucagon, which acts at the translational level via the intermediate cyclic AMP.

With continuous carcinogen treatment the steroid-mediated induction became progressively larger, even though the animals all had massive hepatic tumors and were near death by the end of the experiment. However, the response to glucagon was progressively diminished and was virtually absent in later stages of the experiment. These results are consistent with the conclusion that the induction pathway which is sensitive to glucagon is operating without hormonal stimulation in the livers of the carcinogen-treated rats. This may indicate an effect of the carcinogen on translational processes or that the carcinogen alters cyclic AMP metabolism. These possibilities will be investigated further.

AEC-NIGMS	
Mammalian Comparative Mutagenesis (30.49, 30.50)	**Biological Macromolecular Separations Technology (30.51, 30.52)**
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30.49 STRAIN AND SEX VARIATIONS IN THE SENSITIVITY OF MICE TO DOMINANT-LETHAL INDUCTION WITH ETHYL METHANESULFONATE

W. M. Generoso W. L. Russell[1]

Very little is known about modifying factors that influence mutagenicity of chemicals in mammals. The present experiment, which reveals marked strain and sex differences in sensitivity, points to the need for more information before the results of chemical mutagenicity studies in other test systems are extrapolated to questions of human hazards.

Male and female mice (11 to 13 weeks old) of the strains (SEC × C57BL)F_1, (101 × C3H)F_1, and random-bred T stock were injected intraperitoneally with 300 mg/kg of ethyl methanesulfonate (EMS). The corresponding control mice each received an equal volume (1 ml) of Hanks' balanced salt solution. Treated females were paired individually with (SEC × C57BL)F_1 males 24 hr after injection and separated after the vaginal plugs were observed. Treated males were caged immediately after injection with two (SEC × C57BL)F_1 females (11 to 13 weeks old), and each female which copulated was removed and replaced by a virgin female. All mated females were killed between 13 and 17 days after observation of the vaginal plug and scored for the number of corpora lutea, living embryos, and deciduomata.

Results shown in Table 30.49.1 revealed marked strain variability in the response of female mice to EMS. Of the three strains tested, the T stock exhibited the greatest sensitivity to EMS. The effects of EMS in this strain are expressed as marked reductions in the frequencies of fertile matings and in the average numbers of implants and living embryos in the two intervals. These effects were previously shown by histological examination of ovulated eggs and early cleavage stages as due to induced dominant lethal mutations. In the two hybrids, (SEC × C57BL)F_1 and (101 × C3H)F_1, the EMS-induced frequency of dominant lethals is considerably lower, though still measurable, and there is no significant reduction in the average numbers of implants and living embryos. (This apparent paradox is due to the induced superovulation.) In the (101 × C3H)F_1, a significant decrease in the frequency of fertile matings was found among EMS-treated females mated during the first period. This reduction may have been due to matings being made outside the normal receptive stage of the estrous cycle, when they involved females that were suffering from toxic effects. In the (SEC × C57BL)F_1 strain, the difference in the frequency of fertile matings between EMS-treated and control females was not significant.

Results in the males show all three strains to be sensitive to dominant-lethal induction with EMS (Fig. 30.49.1). The stages in spermatogenesis affected are spermatozoa and, possibly, late spermatids. At these stages EMS caused marked reductions in the average numbers of living embryos, with correspondingly high frequencies of dead implantations. Minor strain dif-

Table 30.49.1. Strain Variation in Sensitivity of Female Mice to Dominant-Lethal Induction with EMS

Strain	Treatment	Treatment to Fertilization Interval (days)	Number of Females	Fertile Matings (%)	Average Number of Corpora Lutea	Average Number of Implants	Average Number of Live Embryos	Induced Dominant Lethals	
								Calculated from Fertile Females Only[a]	Calculated from All Mated Females[b]
T-stock	EMS[c]	1½-4½	69	39.1[d]	11.3	4.6	2.1	69.5	87.2
		5½-9½	34	58.8[d]	10.8	4.8	2.2	66.4	79.2
	Control[e]	1½-4½	65	93.8	11.2	8.5	6.8		
		5½-9½	40	92.5	11.1	8.2	6.7		
(101 × C3H)F_1	EMS	1½-4½	56	83.9[f]	9.4[d]	8.2[d]	7.2	9.7	8.5
		5½-9½	36	88.9	8.8	7.7	6.9	5.4	7.1
	Control	1½-4½	51	96.1	8.1	7.3	6.9		
		5½-9½	42	95.2	8.3	7.3	7.0		
(SEC × C57BL)F_1	EMS	1½-4½	43	74.4	12.2[d]	10.7	9.8	5.2	5.5
		5½-9½	24	79.2	12.1	10.9	9.9	2.1	−12.6
	Control	1½-4½	40	82.5	11.0	9.8	9.3		
		5½-9½	26	73.1	11.4	10.1	9.6		

[a]Calculated using the formula:

$$\%\ \text{D.L.} = \left[1 - \frac{\text{living embryos/corpora lutea (for experimental females)}}{\text{living embryos/corpora lutea (for control females)}} \right] \times 100 .$$

[b]Calculated using the formula:

$$\%\ \text{D.L.} = \left[1 - \frac{\text{living embryos/mating (fertile + "sterile"), experimental}}{\text{living embryos/mating (fertile + "sterile"), control}} \right] \times 100 .$$

[c]All EMS doses were 300 mg per kilogram body weight.

[d]$P < 0.01$.

[e]All controls received 1 ml of Hanks' balanced salt solution.

[f]$P < 0.05$.

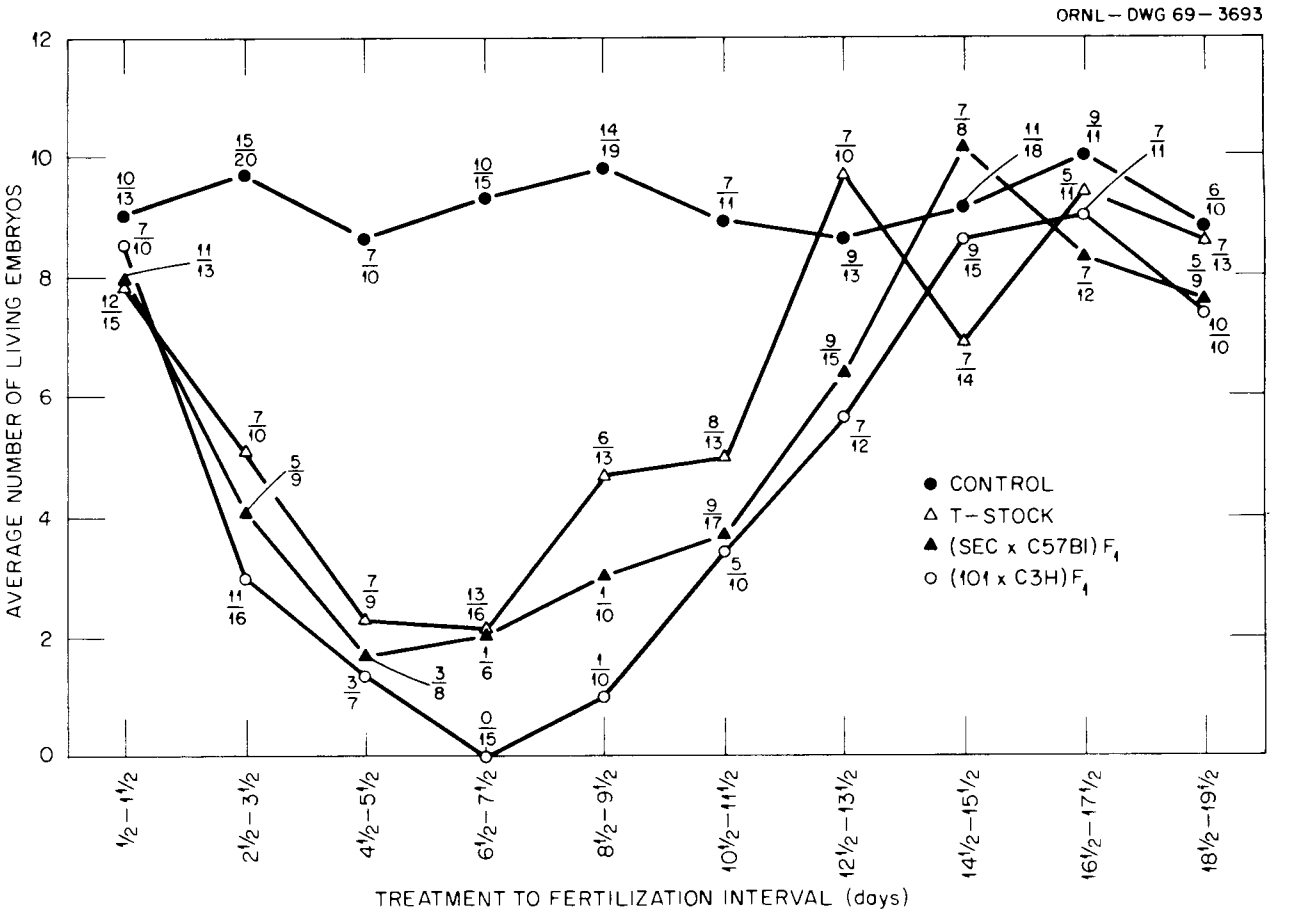

Fig. 30.49.1. Strain Variation in Sensitivity of Male Mice to Dominant-Lethal Induction with EMS. Fraction shown at each point represents the frequency of fertile matings. Control values were obtained by pooling the controls for the three strains.

ferences exist in the sensitivity of male mice to dominant-lethal induction with EMS. In the six intervals included in the sensitive period, 2½ to 13½ days after treatment, T-stock males were slightly but consistently more resistant than were the males of the two hybrids. This is indicated by higher average numbers of living embryos in the six intervals and by the higher frequencies of fertile matings at intervals 6½ to 7½ and 8½ to 9½ days.

In female mice, the T stock exhibited a strong mutagenic action of EMS, while the two hybrids did not. In the case of male mice, all three strains are sensitive to EMS, with only minor strain differences. Or, in other words, while a large sex difference in EMS sensitivity is exhibited by the two hybrid strains, both sexes of the T-stock strain are highly sensitive to dominant-lethal induction with EMS. This finding already shows the complexities that are involved in studying the basic mechanisms of chemical induction of mutations in mammals and in making extrapolations to humans from results obtained in mice and other organisms.

Reference

[1] Mammalian Genetics section.

30.50 A FURTHER STUDY ON CHEMICAL INDUCTION OF DOMINANT LETHALS IN FEMALE MICE

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Eight alkylating compounds are extensively under study for genetic effects in mice: ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), 2-methoxy-6-chloro-9-[3-(ethyl-2-chloroethyl)aminopropylamino] acridine dihydrochloride (ICR-170), triethylene-

melamine (TEM), *n*-propyl methanesulfonate (PMS), isopropyl methanesulfonate (IMS), and 1,4-di(methanesulfonoxy)butane (Myleran). EMS, MMS, MNNG, and ICR-170 have already been tested for induction of dominant lethals in female mice.[1] The present study extends this genetic test to include TEM, IMS, and PMS. Results for Myleran are not yet complete.

T-stock (random-bred) and (101 X C3H)F_1 female mice (11 to 13 weeks old) were injected intraperitoneally with one of the following doses: 75 mg/kg of IMS, 600 or 500 mg/kg of PMS, or 1.6 mg/kg of TEM. These dosages, adjusted for the average body weight, were administered to each animal in 1-ml volumes. Controls received an equal volume of Hanks' balanced salt solution (HBSS). Injected females were mated with (SEC X C57BL)F_1 males at selected intervals during the $\frac{1}{2}$ to 24$\frac{1}{2}$ days test period, killed 13 to 17 days after observation of the vaginal plug, and scored for the number of living embryos and dead implantations.

Results shown in Table 30.50.1 revealed the high mutagenic effects of IMS. In T-stock females, IMS caused marked reductions in the average numbers of implants and live embryos in the first four intervals. These reductions were accompanied by increases in the frequency of dead implantations. The frequency of fertile matings was also markedly reduced during the first three intervals. In general, the effects were higher in the earlier intervals. In (101 X C3H)F_1 females, in which the test has so far been conducted for the first interval only, the effect of IMS is shown as reduced numbers of implants and live embryos and high frequency of dead implants. These effects were, relatively, not as high as those for T stock in the comparable period.

PMS induced low incidence of dominant lethals in T-stock female mice. Slight reductions in the average numbers of implants and embryos and a slight increase in the frequency of dead implants were induced in the first two intervals after treatment with 600 mg/kg. This dose was more toxic to (101 X C3H)F_1 than to T-stock females. Therefore the dose employed in the hybrid strain was reduced to 500 mg/kg, and comparably

Table 30.50.1. Induction of Dominant Lethals with Chemicals in Female Mice

Strain	Compound	Dose (mg/kg)	Treatment to Fertilization Interval (days)	Number of Females	Fertile Matings (%)	Average Number of Implants	Average Number of Live Embryos	Dead Implants (%)
T stock	HBSS (control)	1 ml	$\frac{1}{2}$-4$\frac{1}{2}$	83	92.8	9.4	7.2	23.3
			5$\frac{1}{2}$-9$\frac{1}{2}$	44	95.5	8.0	6.1	23.4
			10$\frac{1}{2}$-14$\frac{1}{2}$	37	97.3	8.6	6.9	20.0
			15$\frac{1}{2}$-19$\frac{1}{2}$	47	97.9	9.2	7.2	21.6
			20$\frac{1}{2}$-24$\frac{1}{2}$	27	96.3	9.2	7.3	20.2
	IMS	75	$\frac{1}{2}$-4$\frac{1}{2}$	36	8.3	4.3	0.3	92.3
			5$\frac{1}{2}$-9$\frac{1}{2}$	36	8.3	4.0	1.0	75.0
			10$\frac{1}{2}$-14$\frac{1}{2}$	29	17.2	7.2	1.4	80.6
			15$\frac{1}{2}$-19$\frac{1}{2}$	34	82.4	6.1	3.3	45.3
			20$\frac{1}{2}$-24$\frac{1}{2}$	34	88.2	9.3	6.7	27.3
	PMS	600	$\frac{1}{2}$-4$\frac{1}{2}$	36	77.8	7.1	4.5	37.5
			5$\frac{1}{2}$-9$\frac{1}{2}$	31	83.9	7.5	5.1	32.0
			10$\frac{1}{2}$-14$\frac{1}{2}$	27	92.6	9.1	6.9	24.6
			15$\frac{1}{2}$-19$\frac{1}{2}$	33	90.9	8.4	6.4	23.7
		500	$\frac{1}{2}$-4$\frac{1}{2}$	35	65.7	8.9	6.4	27.5
	TEM	1.6	$\frac{1}{2}$-4$\frac{1}{2}$	26	84.6	7.5	4.9	34.1
			5$\frac{1}{2}$-9$\frac{1}{2}$	28	89.3	7.4	5.4	27.0
			10$\frac{1}{2}$-14$\frac{1}{2}$	30	93.3	9.2	7.5	19.0
			15$\frac{1}{2}$-19$\frac{1}{2}$	26	92.3	9.7	8.3	14.6
(101 X C3H)F_1	HBSS (control)	1 ml	$\frac{1}{2}$-4$\frac{1}{2}$	60	88.3	7.4	7.0	5.1
	IMS	75	$\frac{1}{2}$-4$\frac{1}{2}$	30	76.7	6.3	3.3	47.9
	PMS	500	$\frac{1}{2}$-4$\frac{1}{2}$	38	63.2	7.9	7.5	5.8
	TEM	1.6	$\frac{1}{2}$-4$\frac{1}{2}$	40	85.0	7.4	5.9	20.6

treated T-stock females were maintained. This dose had no appreciable effect on the frequency of dead implants and average numbers of live embryos in the two strains. Slight reductions in the frequency of fertile matings were noted in both strains, which, very likely, may be due to the toxic effect of the chemical rather than to induced dominant lethals.

The effects of TEM in T-stock females were similar to those of 600 mg/kg of PMS, which induced low incidence of dominant lethals in the first two intervals, as indicated by reduced numbers of implants and living embryos. In the hybrid females, TEM also induced a slight increase in the frequency of dominant lethals, as shown by lower average number of live embryos and higher frequency of dead implants.

So far, of the seven compounds already tested, IMS, PMS, EMS, MMS, and TEM were shown by the dominant-lethal test to cause mutagenic effects in female mice. Large strain differences exist in the sensitivity of female mice to dominant-lethal induction with EMS, MMS, and IMS. IMS and TEM were the only compounds that induced dominant lethals in both strains. IMS, EMS, and MMS are similar in that they induce high frequencies of dominant lethals in T-stock females in this order of effectiveness.

Reference

[1] W. M. Generoso, *Genetics* (in press).

30.51 CHARACTERIZATION OF TWO FORMYL-ACCEPTING SPECIES OF METHIONINE tRNA FROM *ESCHERICHIA COLI* B

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Since the recent report by Kim and Rich[2] indicates that (*E. coli* B) $tRNA_{f1}^{Met}$ can be crystallized, renewed emphasis has been placed on our investigations concerning the chemical, physical, and biological properties of the two $tRNA_f^{Met}$ species reported by Weiss *et al.*[3]

Studies since our last report have revealed the following additional information:

1. No difference can be detected between the two species as determined by melting curve analysis, analytical ultracentrifugation, gel electrophoresis, or Sephadex G-100 column chromatography.
2. Both species contain the minor bases 7-methylguanosine, 4-thiouracil, and 5,6-dihydrouracil. Experiments to determine the exact concentration of these minor bases in each species are being performed.
3. The observed yield of major nucleotides after alkaline hydrolysis is equivalent to the theoretical yield predicted from the primary sequence of $tRNA_f^{Met}$ reported by Dube *et al.*[4]
4. A promising observation is that a formyl-accepting species, chromatographically similar to $tRNA_{f2}^{Met}$, has been observed in unfractionated tRNA's prepared from an RC^{rel} mutant of *E. coli* K_{12}. This is the first time such a species has been observed in tRNA prepared outside the Chemical Technology Division at ORNL.

Most of our observations are consistent with the idea that $tRNA_{f2}^{Met}$ is an artifact that is derived from $tRNA_{f1}^{Met}$ during the large-scale processing or storage of mixed tRNA's.

References

[1] Enzymology section.
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[3] J. F. Weiss, R. L. Pearson, and A. D. Kelmers, *Biochemistry* **7**, 3479 (1968).
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30.52 A SIMPLIFIED PURIFICATION OF $tRNA^{Phe}$ FROM *E. COLI*

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Introduction. – Because of the need for a simple procedure for repurification of individual tRNA's and isolation of undermethylated $tRNA^{Phe}$ from samples containing other undermethylated species, we are devising isolation methods in addition to reversed-phase chromatography.

Results and Discussion. – We have chromatographed the phenoxyacetyl derivative of phenylalanyl-tRNA on BD-cellulose to separate it from other uncharged tRNA's. The discontinuous gradient consists of (1) a low-NaCl gradient, (2) a high-NaCl, ethanol, and Mg^{2+} gradient. Most uncharged tRNA's elute in the low-salt gradient, and the remainder elute early in a separate peak in the second gradient. The derivatized Phe-$tRNA^{Phe}$ elutes last in a separate peak of high specific activity. We are now optimizing conditions for hydrolysis of the phenoxylated Phe-$tRNA^{Phe}$ in order to obtain maximum yields of the purified $tRNA^{Phe}$. This procedure will be applied to the isolation of undermethylated $tRNA^{Phe}$ after reversed-phase chromatography.

Reference

[1] ORNL Chemical Technology Division.

AEC-NASA SPACE BIOLOGY

Space Biology – High-Energy Proton Studies

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Space Biology – Biosatellite

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30.53 THE *NEUROSPORA* EXPERIMENT ON THE GEMINI-XI MISSION

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The *Neurospora* experiment on the Gemini-XI mission utilized spores of a two-component heterokaryon collected on the surface of Millipore filters and in suspension. In a previous report[2] we presented data on the dose-effect curves for survival and the overall forward-mutation frequencies in the *ad-3* region. In brief, these data showed that there was no difference between the dose-response curves for either effect for conidia on Millipore filters. For conidia in suspension, however, the flight samples showed higher levels of survival and lower forward-mutation frequencies in the *ad-3* region than the ground control samples. The detailed genetic analysis of the *ad-3* mutations from both portions of this experiment has now been completed.

The data for the Millipore filter samples show that there is no difference between the spectra of *ad-3* mutations in the flight and ground control samples. (1) No difference was found between the overall dose-effect curves for point mutations or chromosome deletion mutations. (2) When the overall induction curve for point mutations was broken down into its individual components, no difference was found between the dose-response curves for point mutations at

the *ad-3A* locus or the *ad-3B* locus. (3) When the overall induction curve for chromosome deletions was broken down into its individual components, no difference was found between the dose-response curves for deletions covering the *ad-3A* locus, the *ad-3B* locus, or both loci simultaneously. (4) The results of tests for allelic complementation showed that there is no difference between the flight and ground control samples in the dose-response curves for nonpolarized, polarized, or noncomplementing mutants.

The data for the samples in suspension show that the antagonist effect of flight on the overall dose-effect curves for forward mutation in the *ad-3* region is due to a specific reduction in the frequencies of all classes of point mutations. (1) When the overall induction curve for the *ad-3* region was broken down into its two main components, we found significantly lower frequencies of point mutations in the flight samples than the ground control samples, but there was no difference between the frequencies of multilocus deletion mutations. (2) When the overall induction curve for point mutations was broken down into its components, we found lower frequencies of both classes of point mutations at the *ad-3A* locus and at the *ad-3B* locus in the flight samples than in the ground control samples. (3) When the overall induction curve for chromosome deletions was broken down into its individual components, no difference was found between the flight and ground control samples for the dose-response curves for deletions covering the *ad-3A* locus, the *ad-3B* locus, or both loci simultaneously. (4) The results of tests for allelic complementation on *ad-3B* mutants showed that each of the classes of complementation pattern is lower in the flight samples than the ground control samples. These latter data indicate that the reduction in the frequencies of *ad-3B* point mutations obtained with flight has resulted from a comparable reduction in each class of complementation pattern. From this we can conclude that there is no difference between the spectra of genetic alterations at the molecular level in the flight and ground control samples of point mutations.

In summary, the data from the *Neurospora* experiment on the Gemini-XI mission show that the modification of radiation-induced genetic damage by weightlessness is dependent upon the assay system. No effect of weightlessness was found with the metabolically inactive samples on Millipore filters in either the Gemini-XI mission or the Biosatellite-II mission. Antagonistic effects of weightlessness on both survival and mutation induction were found with metabolically active spores in suspension on the Gemini-XI mission. These results are in agreement with other experiments[3] on the Biosatellite-II mission, where both antagonistic and synergistic effects were found with different assay systems.

References

[1] Biometrics and Statistics, Mathematics Division.

[2] F. J. de Serres *et al., Biol. Div. Semiann. Progr. Rept. Jan. 31, 1967*, ORNL-4100, pp. 204–6.

[3] See Biosatellite-II Experiment Symposium, *Bioscience* **18**, 538–661 (1968).

30.54 THE *NEUROSPORA* EXPERIMENT ON THE BIOSATELLITE-II MISSION

F. J. de Serres B. B. Webber

The three-day Biosatellite program was designed primarily to study (1) the effects of weightlessness on basic biological processes and (2) the effects of irradiation under weightlessness on such genetic effects as killing, mutation induction, and the production of chromosome rearrangements. The three-day Biosatellite experiment (Biosatellite-II) was successfully flown in September 1967. The *Neurospora* experiment utilized two different assays to evaluate the genetic effects of weightlessness and weightlessness in combination with radiation. These assays are (1) the inactivation (or killing) of cells and (2) the induction of recessive lethal mutations at specific loci (*ad-3A* and *ad-3B*) resulting both from point mutation and chromosome deletion. The assay system consists of a two-component heterokaryon which is obtained from the fusion of two different haploid strains, each containing a series of genetic markers. The Biosatellite-II *Neurospora* experiment consisted of simultaneously irradiated flight samples and ground control samples. Preflight and postflight ground control experiments using radiation alone or radiation in combination with such space-flight parameters as centrifugation and vibration have also been performed. Any effect of weightlessness should show up as a consistent difference between flight samples and ground control samples.

Present data on the flight samples and all ground-based control data show that there is no effect of weightlessness on radiation-induced genetic effects: (1) No difference was found between the dose-response curves for the in-flight and ground control samples for survival. (2) No difference was found between the frequencies of recessive lethal mutations which occur over the entire genome. (3) No difference was found between the overall dose-response curves for the production of recessive lethal mutations in the *ad-3* region.

The most sensitive assay in the *Neurospora* experiment results from the characterization of the specific locus mutations. The data from these tests enable us to break down the overall induction curves into their individual components, namely, point mutations and chromosome deletions. In addition, these same tests provide a tentative identification of the genetic alterations in the point mutations at the *ad-3B* locus at the molecular level. The characterization of the specific locus mutations in the flight samples, the simultaneous ground control, and the preflight ground control has been completed. Comparison of the dose-response curves for each of the subclasses of recessive lethal mutations has shown that even at this level of organization there is no difference between the flight and ground control samples: (1) No difference was found between the overall induction curves for point mutations or the overall induction curves for chromosome deletion mutations in the *ad-3* region in the flight and ground control samples. (2) When the overall chromosome deletion curve was broken down into its individual components, no difference was found between the flight and ground control samples for the induction of deletions covering the *ad-3A* locus, the *ad-3B* locus, or both loci simultaneously. (3) When the overall induction curve for point mutations was broken down into its individual components, no difference was found between the flight and ground control samples for the induction of point mutations at the *ad-3A* locus or point mutations at the *ad-3B* locus. (4) The results of tests for allelic complementation show that there is no difference between the flight and ground control samples in the dose-response curves for nonpolarized, polarized, or noncomplementing mutants. These latter results clearly show that there is no effect of weightlessness on the spectrum of radiation-induced genetic alterations at the molecular level.

In summary, the data from the study of the induction of recessive lethal mutations in the Biosatellite-II *Neurospora* experiment show that there is no effect of weightlessness on the genetic effects of radiation. In this respect, these data confirm the results obtained with conidial samples on Millipore filters in the *Neurospora* experiment on the Gemini-XI mission. It is important to note that both experiments show that there is no effect of weightlessness on the genetic effects of radiation on metabolically inactive and nondividing biological samples. Both antagonistic and synergistic effects of weightlessness have been found in other experiments[1] on the Biosatellite-II mission utilizing metabolically active or dividing biological samples.

Reference

[1] See Biosatellite-II Experiment Symposium, *Bioscience* **18**, 538–661 (1968).

30.55 THE BIOLOGICAL RESPONSE OF *HABROBRACON* TO SPACE FLIGHT

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The *Habrobracon* experiment was designed to survey a wide spectrum of biological responses to the space environment. The effects of space flight were measured in about 30 mutational, physiological, biochemical, and behavioral end points in this small parasitic wasp. At the five different radiation exposures used, then, the number of absolute end points is in the order of 150. Twenty-two investigators from several different institutions worked together for a limited time before, during, and after the flight so that a maximum amount of information could be obtained from this unique experience.

A detailed description of the experimental design and methods has been published.[4] Two hundred seventy-eight males of the *lemon* mutant strain and females heterozygous for the *lemon, honey,* and *cantaloup* markers were placed into the flight vehicle. The wasps were placed relative to an ^{85}Sr source to receive nominal radiation exposures of 4000, 2000, 1000, 500, and 0 r. Three ground-based controls included: (1) an exact mockup of the spacecraft with a radiation source (control I), (2) an incubator set at constant temperature (control II), and (3) an incubator designed to follow by 1 hr the temperature of the spacecraft (control III).

Table 30.55.1 summarizes the effects of space flight and radiation plus space flight in 16 major end points.

The first observation made after the flight of Biosatellite II was that all the *Habrobracon* survived the flight in excellent condition.

Second, the mating behavior of the males that had been in the spacecraft was altered. Observed matings of 243 males from the ground-based control setup at Cape Kennedy were achieved in about 3 hr by three

Table 30.55.1. Summary of Effects Obtained in the *Habrobracon* Experiment in Biosatellite II

N = null, + = enhancing, – = antagonistic

End Point	Effect	
	Flight	Flight Plus Radiation
Survival of males	N	N
Survival of females	N	N
Mating behavior of males	–	–
Sperm		
Fertilizing capacity	N	+
Dominant lethal mutations	N	N
Recessive lethal mutations	+	N
Translocations	N	N
Xanthine dehydrogenase activity in males	–	–
Oocytes		
Metaphase I: total lethality	+	+
Late prophase I: total lethality	N	N
Late prophase I: recombination	N	N
Early prophase I: total lethality	N	+
Transitional oogonia		
Fecundity	N	–
Hatchability	N	–
Primitive oogonia		
Fecundity	N	–
Hatchability	N	–
Life-span of females	–	–

investigators. At Hickam Field it took three investigators over 13 hr to achieve with certainty one-third of the 254 planned matings of the flight animals. These males from the spacecraft were disoriented and unable to find or copulate with the females. Most of the males must have recovered during the trip from Hickam Field to Oak Ridge, because genetic studies indicated that 80.3% of the females had mated with the males from the spacecraft prior to being set for oviposition. Ninety-three percent of the males from the ground-based control setup mated. This abnormal behavior was not observed in the postflight ground control tests.

The fertilizing capacity of the sperm from the males in the spacecraft was greater than the sperm from males in the ground-based control setup. The increased fertilizing capacity appears to be an enhancing effect of radiation combined with weightlessness, because the males not exposed to irradiation and those exposed to irradiation prior to launch did not show this effect.

There were no significant differences between the flight and ground-based control setup when spontaneous or radiation-induced dominant lethal mutations in sperm were the criterion. A threefold increase in spontaneous recessive lethal mutation frequency was observed in sperm from orbited males when compared with the ground-based control setup (1.8 vs 5.8%). No apparent differences were found between flight and ground controls for radiation-induced recessive lethal mutations or translocations. Since the analysis of the postflight ground control data for radiation-induced recessive lethal mutations and translocations is not completed, the final comparisons cannot yet be made.

Dr. E. C. Keller found a reduced xanthine dehydrogenase activity in the males from the flight vehicle when compared with the ground-based control setup. Similar results were obtained in adult *Drosophila* from Biosatellite II.

Flight conditions enhanced the effect of gamma radiation in producing lethality in metaphase I oocytes. Greater lethality was also obtained in metaphase I oocytes in females from the spacecraft than in the ground control x irradiated prior to launch. It appears that the radiation damage was not repaired as effectively in the spacecraft as in the ground-based control.

Lethality in metaphase I oocytes from nonirradiated females in two of the five modules within the spacecraft was significantly higher than for those in the ground-based control setup. These differences were not observed in the postflight control experiments.

Hatchabilities of eggs exposed to radiation as late prophase I oocytes in the spacecraft and the ground controls were above 90%. Genetic recombination in chromosome I was not affected by radiation nor space flight.

Eggs laid on the third day after the flight responded like metaphase I oocytes in that there was a large radiosensitivity difference between those irradiated in the spacecraft and those on Earth. Eggs from orbited females were more sensitive.

Reproductive performance of females is the principal criterion for effects on transitional and primitive oogonia. The pattern of egg production for *Habrobracon* females recovered from the spacecraft differed drastically from results obtained from females in the ground controls. The ground control females behaved as expected; egg production dropped after the first three days of oviposition to a low period for five to nine days and then increased thereafter, but not to the control level. The females from the spacecraft showed a compensatory pattern of egg production. Although egg production was slightly lower for irradiated flight females than the ground and flight unirradiated controls (0 r) for the first week, egg production was higher than the 0 r controls for the remaining portion of the 20-day study. These results indicate an antagonistic effect of space flight with radiation on oogonia.

Although a comparison of individual dose levels shows few statistical differences, a significantly longer life-span of females from the spacecraft can be shown for pooled data when compared with those of the ground controls.

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