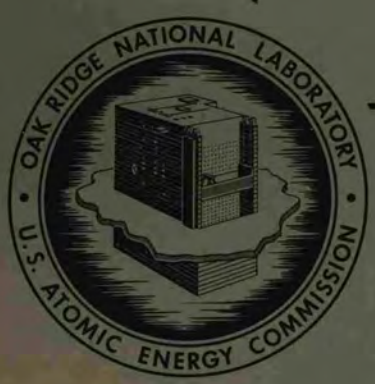
ORNL-4535
Progress

BIOLOGY DIVISION
ANNUAL PROGRESS REPORT
FOR PERIOD ENDING DECEMBER 31, 1969



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

ORNL-4535
Progress

Contract No. W-7405-eng-26

BIOLOGY DIVISION

ANNUAL PROGRESS REPORT

For Period Ending December 31, 1969

H. I. Adler, Director
S. F. Carson, Deputy Director

ISSUED

JUNE 1970

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

Reports previously issued in this series are as follows:

ORNL-13	Period Ending February 29, 1948
ORNL-61	Period Ending May 31, 1948
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ORNL-2593	Period Ending August 15, 1958
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ORNL-2913	Period Ending February 15, 1960
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Contents

Publications

During the period January 1 through December 31, 1969, 504 manuscripts written or edited by members of the Biology Division for publication in the open literature were published or are in press; 3 ORNL reports were issued.

Listed below are the 224 papers, 2 issues of *Experimental Hematology,* 1 authored and 3 coedited books, 1 edited collection of Conference Abstracts, the *Symposium on Protein–Nucleic Acid Interaction,* and 3 ORNL reports published during the period. Not listed are the 73 abstracts and 2 book reviews published or the 197 items currently in press.

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Adler, H. I., W. D. Fisher, and Alice A. Hardigree	Cell division in *Escherichia coli*	*Trans. N.Y. Acad. Sci.* **31**, 1069–70 (1969)
Allen, R. C.	Bi-directional containment marks facility design	*Lab. Management* 7, 38–44 (1969)
Anderson, Louise E., and R. C. Fuller	Photosynthesis in *Rhodospirillum rubrum.* IV. Isolation and characterization of ribulose-1,5-diphosphate carboxylase	*J. Biol. Chem.* **244**, 3105–9 (1969)
Anderson, R. E., R. B. Cumming, Marva F. Walton, and Fred Snyder	Lipid metabolism in cells grown in tissue culture: *O*-alkyl, *O*-alk-1-enyl, and acyl moieties of L-M cells	*Biochim. Biophys. Acta* **176**, 491–501 (1969)
Andrews, G. A., C. C Congdon, C. L. Edwards, Nazareth Gengozian, Bill Nelson, and Helen Vodopick	Preliminary trials of clinical immunotherapy	Pp. 382–86 in *Year Book of Cancer, 1969,* ed. by R. L. Clark and R. W. Cumley, Year Book Medical Publishers, Inc., Chicago, Ill., 1969
Axelrod, D. E., and H. I. Adler	Influence of the fertility episome on the survival of x-irradiated *Escherichia coli*	*J. Bacteriol.* 98, 329–30 (1969)
Barnett, W. E., D. H. Brown, and J. L. Epler	Mitochondrial transfer RNA's and aminoacyl-RNA synthetases	Pp. 100–106 in *Genetics and Developmental Biology,* ed. by H. J. Teas, University of Kentucky Press, Lexington, 1969
Barnett, W. E., C. J. Pennington, Jr., and S. A. Fairfield	Induction of *Euglena* transfer RNA's by light	*Proc. Natl. Acad. Sci. U.S.* **63**, 1261–68 (1969)
Beattie, K. L., and Jane K. Setlow	Killing of *Haemophilus influenzae* cells by integrated ultraviolet-induced lesions from transforming DNA	*J. Bacteriol.* **100**, 1284–88 (1969)
Bender, M. A	Human radiation cytogenetics	Pp. 215–75 in *Advances in Radiation Biology,* vol. 3, ed. by L. G. Augenstein, Ronald Mason, and Max Zelle, Academic, New York and London, 1969
Bender, M. A, and M. A. Barcinski	Kinetics of two-break aberration production by x-rays in human leukocytes	*Cytogenetics* 8, 241–46 (1969)
Bender, M. A, and J. G. Brewen	Factors influencing chromosome aberration yields in the human peripheral leukocyte system	*Mutation Res.* 8, 383–99 (1969)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Bender, M. A, and M. A. Kastenbaum	Statistical analysis of the normal human karyotype	*Am. J. Human Genet.* **21**, 322–51 (1969)
Bennett, Michael, R. A. Steeves, Gustavo Cudkowicz, E. A. Mirand, and Liane B. Russell	Mutant Sl alleles of mice affect susceptibility to Friend spleen focus-forming virus	*Science* **162**, 564–65 (1968)
Bertsch, W. F., Janet West, and Robert Hill	Delayed light studies on photosynthetic energy conversion. II. Effect of electron acceptors and phosphorylation cofactors on the millisecond emission from chloroplasts	*Biochim. Biophys. Acta* **172**, 525–38 (1969)
Boling, M. E., and Jane K. Setlow	Dependence of vegetative recombination among *Haemophilus influenzae* bacteriophage on the host cell	*J. Virology* **4**, 240–43 (1969)
Bollum, F. J.	Deoxynucleotide polymerizing enzymes from calf thymus gland	Pp. 591–611 in *Methods in Enzymology*, ed. by S. P. Colowick and N. O. Kaplan (vol. XII, *Nucleic Acids, Part B*, ed. by Lawrence Grossman and Kivie Moldave), Academic, New York and London, 1968
	Filter paper disk techniques for assaying radioactive macromolecules	*Ibid.*, pp. 169–73
Brewen, J. G., G. Olivieri, H. E. Luippold, and F. G. Pearson	Nonrandom rejoining in the formation of chromatid interchanges: Variations through the cell cycle and the effect of chromosome pairing	*Mutation Res.* 8, 401–8 (1969)
Brewen, J. G., and W. J. Peacock	The effect of tritiated thymidine on sister-chromatid exchange in a ring chromosome	*Mutation Res.* 7, 433–40 (1969)
	Restricted rejoining of chromosomal subunits in aberration formation: A test for subunit dissimilarity	*Proc. Natl. Acad. Sci. U.S.* **62**, 389–94 (1969)
Brick, J. O., R. F. Newell, and D. G. Doherty	A barrier system for a breeding and experimental rodent colony: Description and operation	*Lab. Animal Care* **19**, 92–97 (1969)
Brockman, H. E., F. J. de Serres, and W. E. Barnett	Analysis of *ad-3* mutants induced by nitrous acid in a heterokaryon of *Neurospora crassa*	*Mutation Res.* 7, 307–14 (1969)
Brown, R. C., C. B. Richter, and M. D. Bloomer	Ultrastructural pathology of an acute fatal enteritis of captive cottontail rabbits: Search for a viral etiologic agent	*Am. J. Pathol.* **57**, 93–126 (1969)
Caro, L. G.	Chromosomes of bacteria	Pp. 126–45 in *Handbook of Molecular Cytology*, ed. by A. Lima-de-Faria (vol. 15 in the series *Frontiers of Biology*, general eds. E. L. Tatum and A. Neuberger), North-Holland, Amsterdam and London, 1969
	A common source of difficulty in high-resolution radioautography	*J. Cell Biol.* **41**, 918–19 (1969)
Caro, L. G., and Claire M. Berg	Chromosome replication in *Escherichia coli*. II. Origin of replication in F^- and F^+ strains	*J. Mol. Biol.* **45**, 325–36 (1969)
	Chromosome replication in some strains of *Escherichia coli* K-12	*Cold Spring Harbor Symp. Quant. Biol.* **33**, 559–73 (1968)
Chu, E. H. Y., Patricia Brimer, K. Bruce Jacobson, and E. Virginia Merriam	Mammalian cell genetics. I. Selection and characterization of mutations auxotrophic for L-glutamine or resistant to 8-azaguanine in Chinese hamster cells *in vitro*	*Genetics* **62**, 359–77 (1969)
Chu, E. H. Y., and H. V. Malling	Mammalian cell genetics. II. Chemical induction of specific locus mutations in Chinese hamster cells *in vitro*	*Proc. Natl. Acad. Sci. U.S.* **61**, 1306–12 (1968)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Cohen, Amikam, W. D. Fisher, Roy Curtiss III, and H. I. Adler	The properties of DNA transferred to minicells during conjugation	*Cold Spring Harbor Symp. Quant. Biol.* **33**, 635–41 (1968)
Cohn, W. E.	Representation of macromolecules and polymers of biological importance	*J. Chem. Doc.* 9, 235–41 (1969)
Congdon, C. C (editor)	Experimental Hematology No. 18 (xii + 47 pp.)	Abstracts from the Bone Marrow Conference on Preservation of Hemopoietic, Peripheral Blood, and Lymphatic Tissue Elements held December 5–6, 1968, in New York City (Arthur W. Rowe, chairman); editorial; three contributed papers
	Experimental Hematology No. 19 (viii + 61 pp.)	Abstracts from the Bone Marrow Conference on Bone Marrow Transplantation and Chemical Protection held April 14, 1969, at Atlantic City, New Jersey (James P. OKunewick, chairman); and four contributed papers
Congdon, C. C	Germinal centers, Hassall's corpuscles and epithelioid granulation tissue	Pp. 483–88 in *Advances in Experimental Medicine and Biology*, vol. 5 – *Lymphatic Tissue and Germinal Centers in Immune Response*, ed. by L. Fiore-Donati and M. G. Hanna, Jr., Plenum, New York, 1969
	Lymphatic tissue germinal centers in immune reactions	Pp. 309–37 in *Progress in Biophysics and Molecular Biology*, vol. 19, Part 2, *Biophysics*, ed. by J. A. V. Butler and D. Noble, Pergamon, Oxford-London-Edinburgh-New York-Toronto-Sydney-Paris-Braunschweig, 1969
	Secondary disease in radiation chimeras	Pp. 21–27 in *Bone-Marrow Conservation, Culture, and Transplantation*, Proceedings series, IAEA, Vienna, Austria, 1969
Cook, J. S., and James D. Regan	Photoreactivation and photoreactivating-enzyme activity in an order of mammals (Marsupialia)	*Nature* **223**, 1066–67 (1969)
Cosgrove, G. E., P. B. Dunaway, and J. D. Story	Malignant tumors associated with subcutaneously implanted ^{60}Co radioactive wires in *Peromyscus maniculatus*	*Bull. Wildlife Disease Assoc.* **5**, 311–14 (1969)
Cosgrove, G. E., D. R. Lincicome, and A. A. Warsi	Development of *Trypanosoma lewisi* in experimental chimaeras	*J. Protozool.* **16**, 47–49 (1969)
Cudkowicz, Gustavo	Hybrid resistance to parental grafts of hematopoietic and lymphoma cells	Pp. 661–91 in *The Proliferation and Spread of Neoplastic Cells* (a collection of papers presented at the Twenty-First Annual Symposium on Fundamental Cancer Research held at Houston, Texas, February 27–March 1, 1967), the Williams & Wilkins Company, Baltimore, Md., 1968
Curtiss, Roy, III	Bacterial conjugation	*Ann. Rev. Microbiol.* **23**, 69–136 (1969)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Curtiss, Roy, III, L. G. Caro, D. P. Allison, and D. R. Stallions	Early stages of conjugation in *Escherichia coli*	*J. Bacteriol.* **100**, 1091–1104 (1969)
Curtiss, Roy, III, L. J. Charamella, D. R. Stallions, and Jane Ann Mays	Parental functions during conjugation in *Escherichia coli* K-12	*Bacteriol. Rev.* **32**, 320–48 (1968)
Curtiss, Roy, III, and Janet Renshaw	F^+ strains of *Escherichia coli* K-12 defective in Hfr formation	*Genetics* **63**, 7–26 (1969)
	Kinetics of F transfer and recombinant production in $F^- \times F^+$ matings in *Escherichia coli* K-12	*Ibid.*, pp. 39–52
Curtiss, Roy, III, and D. R. Stallions	Probability of F integration and frequency of stable Hfr donors in F^+ populations of *Escherichia coli* K-12	*Ibid.*, pp. 27–38
Davis, D. G.	Chromosome behavior under the influence of claret-nondisjunctional in *Drosophila melanogaster*	*Genetics* **61**, 577–94 (1969)
de Serres, F. J.	Comparison of the complementation and genetic maps of closely linked nonallelic markers on linkage group I of *Neurospora crassa*	*Mutation Res.* 8, 43–50 (1969)
	Effects of radiation during space flight on microorganisms and plants on the Biosatellite II and Gemini XI	Pp. 62–66 in *(COSPAR) Life Sciences and Space Research VII*, ed. by W. Vishniac and F. G. Favorite, North-Holland, Amsterdam, The Netherlands, and London, 1969
	Genic and chromosomal mutagenesis and nonallelic complementation	*Neurospora Newsletter* **13**, 3–4 (1968)
de Serres, F. J., and H. V. Malling	Identification of the genetic alterations in specific locus mutants at the molecular level	*Japan. J. Genet.* **44** (suppl. 1), 106–13 (1969)
de Serres, F. J., Ida Ruth Miller, Diana B. Smith, Sohei Kondo, and M. A Bender	The Gemini-XI S-4 spaceflight radiation interaction experiment. II. Analysis of survival levels and forward-mutation frequencies in *Neurospora crassa*	*Radiation Res.* 39, 436–44 (1969)
de Serres, F. J., and B. B. Webber	The effect of weightlessness on the induction of recessive lethal mutations in the *Neurospora* experiment on the Biosatellite II mission	ORNL-TM-2566
Doherty, D. G.	Effect of syngeneic bone marrow cell dose on the 30-day and 90-day LD_{50} of x-irradiated mice	*Radiation Res.* 37, 299–304 (1969)
Doherty, D. G., and L. H. Smith	Effect of small doses of syngeneic bone marrow on the 30- and 90-day LD_{50} of x-irradiated mice	*Radiation Res.* **40**, 85–92 (1969)
Dolin, M. I.	Kinetics of malic-lactic transhydrogenase. Abortive complex formation with substrates and products	*J. Biol. Chem.* **244**, 5273–85 (1969)
Dumont, J. N.	Oogenesis in the annelid *Enchytraeus albidus* with special reference to the origin and cytochemistry of yolk	*J. Morphol.* **129**, 317–44 (1969)
Dunn, G. R., T. G. Wilson, and K. Bruce Jacobson	Age-dependent changes in alcohol dehydrogenase in *Drosophila*	*J. Exptl. Zool.* **171**, 185–90 (1969)
Ehling, U. H.	Maternal effect on reproductive capacity of irradiated female mice	*Z. Naturforsch. B* **23**, 1476–78 (1968)
Epler, J. L.	The mitochondrial and cytoplasmic transfer ribonucleic acids of *Neurospora crassa*	*Biochemistry* 8, 2285–90 (1969)

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Finamore, F. J., and J. S. Clegg	Biochemical aspects of morphogenesis in the brine shrimp, *Artemia salina*	Pp. 249–78 in *The Cell Cycle: Gene-Enzyme Interactions (Cell Biology: A Series of Monographs)*, ed. by G. M. Padilla, G. L. Whitson, and I. L. Cameron, Academic, New York and London, 1969
Fiore-Donati, L., and M. G. Hanna, Jr. (editors)	*Advances in Experimental Medicine and Biology*, Vol. 5 – *Lymphatic Tissue and Germinal Centers in Immune Response* (xxiii + 529 pp.)	Plenum, New York, 1969
Fisher, C. R.	Enzymology of the pigmented adenine-requiring mutants of *Saccharomyces* and *Schizosaccharomyces*	*Biochem. Biophys. Res. Commun.* **34**, 306–10 (1969)
	Phosphoribosyl-aminoimidazole-succinocarboxamide synthetase from *Neurospora crassa*. I. Partial purification and properties	*Biochim. Biophys. Acta* **178**, 380–88 (1969)
Fisher, W. D., H. I. Adler, F. W. Shull, Jr., and Amikam Cohen	Properties of a cell fraction that repairs damage to the cell division mechanism of *Escherichia coli*	*J. Bacteriol.* **97**, 500–505 (1969)
Fralick, J. A., W. D. Fisher, and H. I. Adler	Polyuridylic acid-directed phenylalanine incorporation in minicell extracts	*J. Bacteriol.* **99**, 621–22 (1969)
Francis, A. A., and G. L. Whitson	Ultraviolet-induced pyrimidine dimers in *Tetrahymena pyriformis*. II. *In vivo* photoreactivation	*Biochim. Biophys. Acta* **179**, 253–57 (1969)
Fuller, R. C., and N. A. Nugent	Pteridines and the function of the photosynthetic reaction center	*Proc. Natl. Acad. Sci. U.S.* **63**, 1311–18 (1969)
Gassen, H. G.	Separation of oligonucleotides derived from the phenylalanine transfer ribonucleic acid of *Escherichia coli* B by thin-layer techniques	*J. Chromatog.* **39**, 147–56 (1969)
Generoso, W. M.	Chemical induction of dominant lethals in female mice	*Genetics* **61**, 461–70 (1969)
Generoso, W. M., and W. L. Russell	Strain and sex variations in the sensitivity of mice to dominant-lethal induction with ethyl methanesulfonate	*Mutation Res.* **8**, 589–98 (1969)
Gengozian, Nazareth, and C. C Congdon	Effect of syngeneic antilymphocyte serum on rat-mouse radiation chimeras	*Transplant. Proc.* **1**, 34–38 (1969)
Gooch, P. Carolyn, and C. A. Berry	Chromosome analyses of Gemini astronauts	*Aerospace Med.* **40**, 610–14 (1969)
Gooch, P. Carolyn, and C. L. Fisher	High frequency of a specific chromosome abnormality in leukocytes of a normal female	*Cytogenetics* **8**, 1–8 (1969)
Grell, Rhoda F.	Meiosis – Application of basic knowledge to humans	Pp. 27–42 in *Symposium on Human Cytogenetics*, ed. by C. B. Lozzio and A. I. Chernoff, University of Tennessee, 1969
	Meiotic and somatic pairing	Pp. 361–492 in *Genetic Organization – A Comprehensive Treatise*, vol. 1, ed. by E. W. Caspari and A. W. Ravin, Academic, New York and London, 1969
Groves, D. L., W. E. Lever, and Takashi Makinodan	Stochastic model for the production of antibody-forming cells	*Nature* **222**, 95–97 (1969)
Haber, A. H., D. E. Foard, and Stella W. Perdue	Actions of gibberellic and abscisic acids on lettuce seed germination without actions on nuclear DNA synthesis	*Plant Physiol.* **44**, 463–67 (1969)
Haber, A. H., D. E. Foard, and L. L. Triplett	Seedling irradiation of "gamma-plantlets": Demonstration and explanation of a leaf growth stimulation	*Radiation Botany* **9**, 473–79 (1969)
Haber, A. H., and Barbara E. Rothstein	Radiosensitivity and rate of cell division: "Law of Bergonié and Tribondeau"	*Science* **163**, 1338–39 (1969)

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Haber, A. H., and Patricia L. Walne	Actions of acute gamma radiation on excised wheat leaf tissue. I. Radioresistance of senescence and establishing criteria of death from studies of chlorophyll loss	*Radiation Botany* **8**, 389–97 (1968)
Hamkalo, Barbara A., and P. A. Swenson	Effects of ultraviolet radiation on respiration and growth in radiation-resistant and radiation-sensitive strains of *Escherichia coli* B	*J. Bacteriol.* **99**, 815–23 (1969)
Hanna, M. G., Jr., and Mary W. Francis	Specific inhibition of immunocompetence	*Nature* **223**, 1161–62 (1969)
Hanna, M. G., Jr., Paul Nettesheim, and Mary W. Francis	Requirement for continuous antigenic stimulation in the development and differentiation of antibody-forming cells: The effect of passive antibody on the primary and secondary responses	*J. Exptl. Med.* **129**, 953–71 (1969)
Hanna, M. G., Jr., Paul Nettesheim, and H. E. Walburg, Jr.	A comparative study of the immune reaction in germfree and conventional mice	Pp. 237–48 in *Advances in Experimental Medicine and Biology*, vol. 3 – *Germfree Biology: Experimental and Clinical Aspects* (Proceedings of an International Symposium on Gnotobiology held in Buffalo, New York, June 9–11, 1968), ed. by E. A. Mirand and Nathan Back, Plenum, New York, 1969
Hanna, M. G., Jr., A. K. Szakal, and H. E. Walburg, Jr.	The relation of antigen and virus localization to the development and growth of lymphoid germinal centers	Pp. 149–65 in *Advances in Experimental Medicine and Biology*, vol. 5 – *Lymphatic Tissue and Germinal Centers in Immune Response*, ed. by L. Fiore-Donati and M. G. Hanna, Jr., Plenum, New York, 1969
Hellman, Kiki B., Alfred Hellman, R. B. Cumming, and G. David Novelli	Comparative physiology of L-M cells propagated *in vitro* and in female and male mice	*J. Natl. Cancer Inst.* **41**, 653–64 (1968)
Hollaender, Alexander	Foreword (to the Proceedings of the Symposium on Nuclear Physiology and Differentiation)	Pp. iii–iv in *Nuclear Physiology and Differentiation* (Proceedings of an International Symposium held at Belo Horizonte, Minas Gerais, Brazil, December 1–6, 1968), ed. by R. P. Wagner and Esther A. Eakin. (Printed as a supplement to *Genetics*, vol. 61, No. 1), Genetics Society of America, Austin, Tex., 1969
Hollaender, Alexander, and D. G. Doherty	The background for modification of radiation damage by sulfur compounds	Pp. 1–14 in *Radiation Damage and Sulphydryl Compounds.* Proceedings series, IAEA, Vienna, Austria, 1969
Holten, Virginia Z., and K. Bruce Jacobson	Studies on the aminoacylation of valine- and alanine-specific transfer RNA of *E. coli* by aminoacyl transfer RNA synthetases from *Neurospora crassa* and *E. coli*	*Arch. Biochem. Biophys.* **129**, 283–89 (1969)
Horn, E. C., and Marilyn S. Kerr	The hemolymph proteins of the blue crab, *Callinectes sapidus*. I. Hemocyanins and certain other major protein constituents	*Comp. Biochem. Physiol.* **29**, 493–508 (1969)
Jacobson, K. Bruce, J. B. Murphy, and P. B. Dunaway	Phylogenetic relationships of rodents on the basis of isoenzymes of lactate dehydrogenase	*Comp. Biochem. Physiol.* **28**, 1135–44 (1969)

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Jagger, John, D. M. Prescott, and Mary Esther Gaulden	An ultraviolet microbeam study of the roles of nucleus and cytoplasm in division delay, killing, and photoreactivation of *Amoeba proteus*	*Exptl. Cell. Res.* **58**, 35–54 (1969)
Jagger, John, R. S. Stafford, and Jeanne M. Snow	Thymine-dimer and action-spectrum evidence for indirect photoreactivation in *Escherichia coli*	*Photochem. Photobiol.* **10**, 383–95 (1969)
Jakob, K. M., and Sheldon Wolff	The effect of post-irradiation centrifugation on chromosome aberrations in *Vicia faba*	*Intern. J. Radiation Biol.* **15**, 519–23 (1969)
Jared, D. W., and R. A. Wallace	Protein uptake *in vitro* by amphibian oocytes	*Exptl. Cell Res.* **57**, 454–57 (1969)
Jenkins, V. K., A. C. Upton, and T. T. Odell, Jr.	Differences between exogenous and endogenous hemopoietic spleen colonies	*J. Cell. Physiol.* **73**, 141–48 (1969)
	Effect of estradiol on splenic repopulation by endogenous and exogenous hemopoietic cells in irradiated mice	*J. Cell. Physiol.* **73**, 149–57 (1969)
Kenney, F. T., J. R. Reel, C. B. Hager, and J. L. Wittliff	Hormonal induction and repression	Pp. 119–42 in *Regulatory Mechanisms for Protein Synthesis in Mammalian Cells*, ed. by Anthony San Pietro, M. R. Lamborg, and F. T. Kenney, Academic, New York and London, 1968
Kerr, Marilyn S.	The hemolymph proteins of the blue crab, *Callinectes sapidus*. II. A lipoprotein serologically identical to oocyte lipovitellin	*Develop. Biol.* **20**, 1–17 (1969)
Kimball, R. F.	Chairman's opening remarks	Pp. 1–2 in *Mutation as Cellular Process* (A Ciba Foundation Symposium), ed. by G. E. W. Wolstenholme and Maeve O'Connor, J. & A. Churchill Ltd., London, 1969
	The relation between repair of radiation damage and mutation induction	*Photochem. Photobiol.* **8**, 515–20 (1968)
	Studies on mutations induced by ultraviolet radiation in *Paramecium aurelia* with special emphasis on photoreversal	*Mutation Res.* **8**, 79–89 (1969)
	Symposium on Protein–Nucleic Acid Interaction (and Introduction)	*J. Cellular Physiol.* **74**, suppl. 1, October 1969, 266 pp. incl. index
Kimball, R. F., and S. F. Carson	Biology Division Annual Progress Report for the Period Ending December 31, 1968	ORNL-4412
Knopp, J. A., J. J. ten Bosch, and J. W. Longworth	Energy transfer in poly-L-tyrosine. V. Fluorescence polarization studies	*Biochim. Biophys. Acta* **188**, 185–95 (1969)
Kretchmar, A. L.	Comment on cover	*Exptl. Hematol.* **19**, v–vi (1969)
Kretchmar, A. L., and W. R. Conover	Early proliferation of transplanted spleen colony-forming cells. II. Circulation of cells	*Proc. Soc. Exptl. Biol. Med.* **131**, 703–6 (1969)
Kretchmar, A. L., and E. J. Price	Use of respiration pattern analysis for study of serine metabolism *in vivo*	*Metabolism* **18**, 684–91 (1969)
Kull, F. J., and K. Bruce Jacobson	Multiple phenylalanyl-transfer ribonucleic acid synthetase activities in the cytoplasm of *Neurospora crassa*	*Proc. Natl. Acad. Sci. U.S.* **62**, 1137–44 (1969)
Kull, F. J., P. O. Ritter, and K. Bruce Jacobson	Effects of inorganic pyrophosphate on *Neurospora crassa* phenylalanine transfer ribonucleic acid ligase in heterologous aminoacylation reactions	*Biochemistry* **8**, 3015–23 (1969)

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Leibo, S. P., and Peter Mazur	Freezing of bacteriophage T4: Temperature and rate effects as a function of salt concentration	*Virology* **38**, 558–66 (1969)
Lindsley, D. L., and E. H. Grell	Spermiogenesis without chromosomes in *Drosophila melanogaster*	Pp. 69–78 in *Nuclear Physiology and Differentiation* (Proceedings of an International Symposium held at Belo Horizonte, Minas Gerais, Brazil, December 1–6, 1968), ed. by R. P. Wagner and Esther A. Eakin (Printed as a supplement to *Genetics*, vol. 61, No. 1), Genetics Society of America, Austin, Tex., 1969
Longworth, J. W.	Techniques for measuring fluorescence and phosphorescence of biological materials	*Photochem. Photobiol.* 8, 589–99 (1968)
Longworth, J. W., J. J. ten Bosch, J. A. Knopp, and R. O. Rahn	Electronic energy transfer in oligomers and polymers of L-tyrosine	Pp. 529–50 in *Molecular Luminescence*, ed. by E. C. Lim, W. A. Benjamin, Inc., New York and Amsterdam, 1969
Macy, R. L., and P. W. Whiting	Tetraploid females in *Mormoniella*	*Genetics* **61**, 619–30 (1969)
Makinodan, Takashi, W. J. Peterson, J. F. Albright, E. H. Perkins, Fumitoshi Chino, J. M. Ellis, R. P. Quinn, and G. B. Price	Immunological aspects of aging	Pp. 360–63 in *Proceedings of the 8th International Congress of Gerontology*, vol. 1, *Abstracts of Symposia and Lectures*, Federation of American Societies for Experimental Biology, Washington, D.C., 1969
Makinodan, Takashi, Toshihiko Sado, D. L. Groves, and G. B. Price	Growth patterns of antibody-forming cell populations	Pp. 81–113 in *Current Topics in Microbiology and Immunology*, vol. 49, ed. by W. Arber *et al.*, Springer-Verlag, Berlin-Heidelberg-New York, 1969
Malling, H. V., and F. J. de Serres	Mutagenicity of alkylating carcinogens	*Ann. N.Y. Acad. Sci.* **163**, 788–800 (1969)
Mattingly, Ellen, and Shirley Ogle	A new culture method for *Rhynchosciara*	*Ann. Entomol. Soc. Amer.* **62**, 94–96 (1969)
Mazur, Peter	Freezing injury in plants	*Ann. Rev. Plant Physiol.* **20**, 419–48 (1969)
	Physical and chemical changes during freezing and thawing of cells, with special reference to blood cells	Pp. 764–77 in *Proceedings of the Eleventh Congress of the International Society of Blood Transfusion* (Sydney, August 24–29, 1966), Part 3. *Problems in Blood Transfusion. Blood and Bone Marrow Preservation*, ed. by L. Holländer, S. Karger, Basel and New York, 1968
Mazur, Peter, John Farrant, S. P. Leibo, and E. H. Y. Chu	Survival of hamster tissue culture cells after freezing and thawing: Interactions between protective solutes and cooling and warming rates	*Cryobiology* **6**, 1–9 (1969)
McDevitt, D. S., Isaura Meza, and Tuneo Yamada	Immunofluorescence localization of the crystallins in amphibian lens development, with special reference to the γ-crystallins	*Develop. Biol.* **19**, 581–607 (1969)
McDonald, T. P., D. H. Martin, M. L. Simmons, and R. D. Lange	Preliminary results of erythropoietin production by bovine kidney cells in culture	*Life Sciences* **8**, 949–54 (1969)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
McLeish, John	Changes in the amounts of nuclear RNA during interphase in *Vicia faba*	*Chromosoma* **26**, 312–25 (1969)
Mead, C. G., and F. J. Finamore	The occurrence of ascorbic acid sulfate in the brine shrimp, *Artemia salina*	*Biochemistry* 8, 2652–55 (1969)
Miller, O. L., Jr., and Barbara R. Beatty	Extrachromosomal nucleolar genes in amphibian oocytes	Pp. 133–43 in *Nuclear Physiology and Differentiation* (Proceedings of an International Symposium held at Belo Horizonte, Minas Gerais, Brazil, December 1–6, 1968), ed. by R. P. Wagner and Esther A. Eakin (Printed as a supplement to *Genetics,* vol. 61, No. 1), Genetics Society of America, Austin, Tex., 1969
	Nucleolar structure and function	Pp. 605–19 in *Handbook of Molecular Cytology,* ed. by A. Lima-de-Faria (vol. 15 in the series *Frontiers of Biology,* general eds. E. L. Tatum and A. Neuberger), North-Holland, Amsterdam and London, 1969
	Portrait of a gene	*J. Cell. Physiol.* **74** (suppl. 1), 225–32 (1969)
	Visualization of nucleolar genes	*Science* **164**, 955–57 (1969)
Modak, S. P., and Jane K. Setlow	Synthesis of deoxyribonucleic acid after ultraviolet irradiation of sensitive and resistant *Haemophilus influenzae*	*J. Bacteriol.* 98, 1195–98 (1969)
Modak, S. P., R. C. von Borstel, and F. J. Bollum	Terminal lens cell differentiation. II. Template activity of DNA during nuclear degeneration	*Exptl. Cell Res.* **56**, 105–12 (1969)
Montour, J. L.	Radiation-induced involution of the lymphatic organs of the young chicken	*Acta Radiol.* 7, 337–45 (1968)
Moseley, B. E. B.	Repair of ultraviolet radiation damage in sensitive mutants of *Micrococcus radiodurans*	*J. Bacteriol.* 97, 647–52 (1969)
Nettesheim, Paul, and M. G. Hanna, Jr.	Radiosensitivity of the antigen-trapping mechanism and its relation to the suppression of immune response	Pp. 167–75 in *Advances in Experimental Medicine and Biology,* vol. 5 – *Lymphatic Tissue and Germinal Centers in Immune Response,* ed. by L. Fiore-Donati and M. G. Hanna, Jr., Plenum, New York, 1969
Nettesheim, Paul, Mary Lou Williams, and Anna S. Hammons	Regenerative potential of immunocompetent cells. III. Recovery of primary antibody-forming potential from x-irradiation. The role of the thymus	*J. Immunol.* **103**, 505–18 (1969)
Niyogi, S. K.	The influence of chain length and base composition on the specific association of oligoribonucleotides with denatured DNA	*J. Biol. Chem.* **244**, 1576–81, (1969)
Niyogi, S. K., and Pamela E. Wilton	Effect of temperature on the oligonucleotide stimulation of the RNA polymerase reaction	*J. Mol. Biol.* **41**, 149–53 (1969)
Novelli, G. David	The separation of isoaccepting transfer RNA's and the possible role of tRNA in regulation	*J. Cell. Physiol.* **74** (suppl. 1), 121–48 (1969)
O'Dell, D. H., and D. E. Foard	Presence of lateral root primordia in the radicle of buckwheat embryos	*Bull. Torrey Botan. Club* **96**, 1–3 (1969)
Odell, T. T., Jr. (chairman)	Abstracts: Fourth Conference on Blood Platelets	*Blood* **34**, 521–45 (1969)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Odell, T. T., Jr., E. A. Burch, Jr., C. W. Jackson, and T. J. Friday	Megakaryocytopoiesis in mice	*Cell Tissue Kinet.* **2**, 363–67 (1969)
Odell, T. T., Jr., C. W. Jackson, T. J. Friday, and Deborah E. Charsha	Effects of thrombocytopenia on megakaryocytopoiesis	*Brit. J. Haematol.* **17**, 91–101 (1969)
Odell, T. T., Jr., C. W. Jackson, and Rebecca S. Reiter	Generation cycle of rat megakaryocytes	*Exptl. Cell Res.* **53**, 321–28 (1968)
Olins, D. E.	The interaction of lysine-rich histones and DNA	*J. Mol. Biol.* **43**, 439–60 (1969)
Ortwerth, B. J., and G. David Novelli	Studies on the incorporation of L-ethionine-ethyl-1-^{14}C into the transfer RNA of rat liver	*Cancer Res.* **29**, 380–90 (1969)
Padilla, G. M., G. L. Whitson, and I. L. Cameron (editors)	*The Cell Cycle: Gene-Enzyme Interactions* (Cell Biology: A Series of Monographs) (xvii + 399 pp.)	Academic, New York and London, 1969
Pal, B. C., Mayo Uziel, D. G. Doherty, and W. E. Cohn	Isolation and characterization of a pyrimidine sulfenic acid *via* scission of the sulfur–sulfur bond in the methyl analog of bis(4-thiouridine) disulfide	*J. Am. Chem. Soc.* **91**, 3634–38 (1969)
Palmer, Winifred G., and John Papaconstantinou	Biochemical properties of α-crystallins during lens development	Pp. 165–86 in *Differentiation and Immunology*, ed. by Katherine Brehme Warren (Symposia of the International Society for Cell Biology, vol. 7), Academic, New York and London, 1968
Parker, D. R.	Heterologous interchange at meiosis in *Drosophila*. II. Some disjunctional consequences of interchange	*Mutation Res.* **7**, 393–407 (1969)
	On the sequence of elements in compound-XY #129-16 and in some of its derivative marked Y chromosomes	*Drosophila Info. Serv.* **43**, 156 (1968)
Pearlstein, R. M.	Donor fluorescence as a probe of energy transfer	*Photochem. Photobiol.* **8**, 341–47 (1968)
Perkins, E. H., Toshihiko Sado, and Takashi Makinodan	Recruitment and proliferation of immunocompetent cells during the log phase of the primary antibody response	*J. Immunol.* **103**, 668–78 (1969)
Popp, Diana M.	*Histocompatibility*-14; correlation of the isoantigen rho and *R-Z* locus	*Transplantation* **7**, 233–41 (1969)
Popp, R. A.	Studies on the mouse hemoglobin loci. VIII. A fourth α-chain phenotype	*J. Heredity* **60**, 126–28 (1969)
	Studies on the mouse hemoglobin loci. IX. A fifth α-chain phenotype	*J. Heredity* **60**, 128–31 (1969)
	Studies on the mouse hemoglobin loci. X. Linkage of duplicate genes at the α-chain locus, *Hba*	*J. Heredity* **60**, 131–33 (1969)
Popp, R. A., Diana M. Popp, N. G. Anderson, and L. H. Elrod	Use of the zonal centrifuge to separate particles containing transplantation antigen	*Biochim. Biophys. Acta* **184**, 625–33 (1969)
Rahn, R. O.	Electron spin resonance studies of the triplet states of monoanionic thymine	*Photochem. Photobiol.* **9**, 527–30 (1969)
Rahn, R. O., and J. L. Hosszu	Influence of relative humidity on the photochemistry of DNA films	*Biochim. Biophys. Acta* **190**, 126–31 (1969)
	Photochemical studies of thymine in ice	*Photochem. Photobiol.* **10**, 131–37 (1969)
Rahn, R. O., Jane K. Setlow and J. L. Hosszu	Ultraviolet inactivation and photoproducts of transforming DNA irradiated at low temperatures	*Biophys. J.* **9**, 510–17 (1969)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
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Reese, D. H., Egidio Puccia, and Tuneo Yamada	Activation of ribosomal RNA synthesis in initiation of Wolffian lens regeneration	*J. Exptl. Zool.* **170**, 259–68 (1969)
Regan, James D., Helen Vodopick, Susumu Takeda, W. H. Lee, and F. M. Faulcon	Serine requirement in leukemic and normal blood cells	*Science* **163**, 1452–53 (1969)
Richter, C. B., and R. L. Hendren	The pathology and epidemiology of acute enteritis in captive cottontail rabbits (*Sylvilagus floridanus*)	*Pathol. Vet.* 6, 159–75 (1969)
Riggsby, W. S.	A general method for fractionation of nucleic acids on the basis of sequence homology	*Biochemistry* 8, 222–30 (1969)
Ritter, P. O., F. J. Kull, and K. Bruce Jacobson	Effects of Tris and dimethylsulfoxide on the amino-acylation of *Escherichia coli* valine transfer RNA by *Neurospora crassa* phenylalanyl transfer RNA synthetase	*Biochim. Biophys. Acta* **179**, 524–26 (1969)
Robie, D. M., and H. E. Walburg, Jr.	Ethylene-oxide sterilization of plastic-film isolators: A six-year study of operational efficiency	Pp. 357–65 in *Advances in Experimental Medicine and Biology,* vol. 3 – *Germfree Biology: Experimental and Clinical Aspects* (Proceedings of an International Symposium on Gnotobiology held in Buffalo, New York, June 9–11, 1968), ed. by E. A. Mirand and Nathan Back, Plenum, New York, 1969
Rogers, Stanfield	Significance of dialysis against enzymes to the specific therapy of cancer and genetic deficiency diseases	*Nature* **220**, 1321–22 (1968)
	Some views concerning carcinogenesis	Pp. 39–44 in *Proceedings of the University of Kentucky Tobacco and Health Workshop,* University of Kentucky Press, 1968
Russell, Liane B., and Clyde S. Montgomery	Comparative studies on X-autosome translocations in the mouse. I. Origin, viability, fertility, and weight of five T(X;1)'s	*Genetics* **63**, 103–20 (1969)
Russell, W. L.	Observed mutation frequency in mice and the chain of processes affecting it	Pp. 216–28 in *Mutation as Cellular Process* (A Ciba Foundation Symposium), ed. by G. E. W. Wolstenholme and Maeve O'Connor, J. & A. Churchill Ltd., London, 1969
Sado, Toshihiko	Functional and ultrastructural studies of antibody-producing cells exposed to 10,000 r in Millipore diffusion chambers	*Intern. J. Radiation Biol.* **15**, 1–22 (1969)
Sandler, L., D. L. Lindsley, Benedetto Nicoletti, and G. Trippa	Mutants affecting meiosis in natural populations of *Drosophila melanogaster*	*Genetics* **60**, 525–58 (1968)
San Pietro, Anthony, M. R. Lamborg, and F. T. Kenney (editors)	*Regulatory Mechanisms for Protein Synthesis in Mammalian Cells* (477 pp.)	Academic, New York and London, 1968

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Schnall, B. S., and L. H. Smith	A caging effect: Variation in splenic ^{59}Fe uptake resulting from regrouping male mice	*Exptl. Hematol.* 18, 44–47 (1969)
Self, J. T., and G. E. Cosgrove	Pentastome larvae in laboratory primates	*J. Parasitol.* 54, 969 (1968)
Setlow, Jane K., M. L. Randolph, M. E. Boling, Alice Mattingly, G. B. Price, and M. P. Gordon	Repair of DNA in *Haemophilus influenzae*. II. Excision, repair of single-strand breaks, defects in transformation, and host-cell modification in uv-sensitive mutants	*Cold Spring Harbor Symp. Quant. Biol.* 33, 209–18 (1968)
Setlow, R. B.	Alexander Hollaender	*Photochem. Photobiol.* 8, 511–13 (1968)
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Shearer, G. M., Gustavo Cudkowicz, and H. E. Walburg, Jr.	Hemolytic plaque-forming cells and antigen-sensitive units in spleens of germfree and conventional mice	Pp. 269–75 in *Advances in Experimental Medicine and Biology*, vol. 3 – *Germfree Biology: Experimental and Clinical Aspects* (Proceedings of an International Symposium on Gnotobiology held in Buffalo, New York, June 9–11, 1968), ed. by E. A. Mirand and Nathan Back, Plenum, New York, 1969
Shugart, L. R., Barbara H. Chastain, and G. David Novelli	A chromatographically different form of the formyl-accepting methionine transfer RNA from *Escherichia coli*	*Biochem. Biophys. Res. Commun.* 37, 305–12 (1969)
	Use of reversed-phase column chromatography for rapid isolation and identification of formyl-methionyl-tRNA	*Biochim. Biophys. Acta* 186, 384–86 (1969)
Shugart, L. R., and M. P. Stulberg	Borohydride reduction of phenylalanine transfer ribonucleic acid. Effect on enzyme recognition	*J. Biol. Chem.* 244, 2806–8 (1969)
Skinner, Dorothy M.	Deoxyribonucleic acid sequences complementary to ribosomal ribonucleic acid in a crustacean	*Biochemistry* 8, 1467–73 (1969)
Smith, Diana B., and F. J. de Serres	Computer programs for statistical analysis of forward-mutation experiments at specific loci in *Neurospora crassa*	ORNL-TM-2544
Smith, L. H., and T. W. McKinley, Jr.	Protection of mice against radiation injury by phenylhydrazine	*Proc. Soc. Exptl. Biol. Med.* 129, 702–5 (1968)
Smith, L. H., and Henrianne G. Willard	Alteration of hemopoietic tissue as a factor influencing radiosensitivity of the mouse	*Am. J. Physiol.* 216, 493–98 (1969)
Smith, Roger H.	Induction of mutations in *Habrobracon* sperm with mitomycin C	*Mutation Res.* 7, 231–34 (1969)
Spaulding, J. F., Diana M. Popp, and R. A. Popp	A within-strain difference in radiation sensitivity in the RFM mouse	*Radiation Res.* 40, 37–45 (1969)
Stevens, Audrey L.	Studies of DNA-dependent RNA polymerase	*J. Cell. Physiol.* 74 (suppl. 1), 205–18 (1969)
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Stine, G. J.	Enzyme activities during the asexual cycle of *Neurospora crassa.* III. Nicotinamide adenosine diphosphate glycohydrolase	*Can. J. Microbiol.* **15**, 1249–54 (1969)
	Investigations during phases of synchronous development and differentiation in *Neurospora crassa*	Pp. 119–39 in *The Cell Cycle: Gene-Enzyme Interactions (Cell Biology: A Series of Monographs)*, ed. by G. M. Padilla, G. L. Whitson, and I. L. Cameron, Academic, New York and London, 1969
	Nicotinamide-adenine dinucleotidase activity during germination and growth of *Neurospora crassa*	*Can. J. Microbiol.* **15**, 1249–54 (1969)
Stulberg, M. P., K. R. Isham, and Audrey Stevens	An analysis *in vivo* of histidine transfer RNA during repression and derepression in *Bacillus subtilis*	*Biochim. Biophys. Acta* **186**, 297–304 (1969)
ten Bosch, J. J., and J. A. Knopp	Energy transfer in poly-L-tyrosine as a function of the degree of ionization of the phenolic hydroxyls. IV. Calculations of theoretical transfer rates	*Biochim. Biophys. Acta* **188**, 173–84 (1969)
ten Bosch, J. J., J. W. Longworth, and R. O. Rahn	Energy transfer in poly-L-tyrosine as a function of the degree of ionization of the phenolic hydroxyls. III. Luminescence studies at 77°K	*Biochem. Biophys. Acta* **175**, 10–19 (1969)
Tennant, R. W., K. R. Layman, and R. E. Hand, Jr.	Effect of cell physiological state on infection by rat virus	*Virology* **4**, 872–78 (1969)
Upton, A. C.	Effects of radiation on man	*Ann. Rev. Nucl. Sci.* **18**, 495–528 (1968)
	Gerontological significance of studies on the effects of radiation	Pp. 379–81 in *Proceedings of the 8th International Congress of Gerontology*, vol. 1, *Abstracts of Symposia and Lectures*, Federation of American Societies for Experimental Biology, Washington, D.C., 1969
	Radiation Injury: Principles and Perspectives (x + 126 pp.)	The University of Chicago Press, Chicago and London, 1969
Uziel, Mayo, and H. G. Gassen	Phenylalanine transfer ribonucleic acid from *Escherichia coli* B. Isolation and characterization of oligonucleotides from ribonuclease T_1 and ribonuclease A hydrolyzates	*Biochemistry* **8**, 1643–55 (1969)
Uziel, Mayo, and J. X. Khym	Sequential degradation of nucleic acids. Degradation of *Escherichia coli* B phenylalanine tRNA	*Biochemistry* **8**, 3254–60 (1969)
Uziel, Mayo, C. Koh, and W. E. Cohn	Rapid ion-exchange chromatographic microanalysis of ultraviolet-absorbing materials and its application to nucleosides	*Anal. Biochem.* **25**, 77–98 (1968)
Vann, D. C.	*In vitro* antibody synthesis by diffusion chamber culture of spleen cells. II. Effect of increased levels of free antibody	*J. Immunol.* **102**, 451–56 (1969)
Vann, D. C., and Takashi Makinodan	*In vitro* antibody synthesis by diffusion chamber culture of spleen cells. I. Methods and effect of 10,000 r on antibody synthesis	*J. Immunol.* **102**, 442–50 (1969)
Van Pelt, A. F., Katherine Cain, and Robert Knorr	A new hairloss mutant in the house mouse	*J. Heredity* **60**, 78, 96 (1969)

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Volkin, Elliot, and W. S. Riggsby	Some properties of bacteriophage messenger RNA's	Pp. 409–28 in *Nuclear Physiology and Differentiation* (Proceedings of an International Symposium held at Belo Horizonte, Minas Gerais, Brazil, December 1–6, 1968), ed. by R. P. Wagner and Esther A. Eakin (Printed as a supplement to *Genetics,* vol. 61, No. 1), Genetics Society of America, Austin, Tex., 1969
von Borstel, R. C.	On the origin of spontaneous mutations	*Japan. J. Genet.* 44 (suppl. 1), 102–5 (1969)
	The theory of dominant lethality	Pp. 71–73 in *Advances in Insect Population Control by the Sterile-Male Technique,* ed. by G. C. LaBrecque and J. C. Keller, Technical Reports Series No. 44, International Atomic Energy Agency, Vienna, 1965
	Yeast genetics	*Science* **163**, 962–64 (1969)
von Borstel, R. C., O. L. Miller, Jr., and F. J. Bollum	Probing the structure of chromosomes with DNA polymerase and terminal transferase	Pp. 401–8 in *Nuclear Physiology and Differentiation* (Proceedings of an International Symposium held at Belo Horizonte, Minas Gerais, Brazil, December 1–6, 1968), ed. by R. P. Wagner and Esther A. Eakin (Printed as a supplement to *Genetics,* vol. 61, No. 1), Genetics Society of America, Austin, Tex., 1969
von Borstel, R. C., and Crodowaldo Pavan	Alexander Hollaender	*Ibid.,* pp. vii–viii
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 responses of insects in the Biosatellite II experiment	Pp. 70–76 in *(COSPAR) Life Sciences and Space Research VII,* ed. by W. Vishniac and F. G. Favorite, North-Holland, Amsterdam, The Netherlands, and London, 1969
Walburg, H. E., Jr., and G. E. Cosgrove	Reticular neoplasms in irradiated and unirradiated germfree mice	Pp. 135–41 in *Advances in Experimental Medicine and Biology,* vol. 3 – *Germfree Biology: Experimental and Clinical Aspects* (Proceedings of an International Symposium on Gnotobiology held in Buffalo, New York, June 9–11, 1968), ed. by E. A. Mirand and Nathan Back, Plenum, New York, 1969
	Severity of the parent to F_1 graft-versus-host reaction in irradiated germfree mice	*Ibid.,* pp. 277–86
Wallace, R. A., and D. W. Jared	Studies on amphibian yolk. VIII. The estrogen-induced hepatic synthesis of a serum lipophosphoprotein and its selective uptake by the ovary and transformation into yolk platelet proteins in *Xenopus laevis*	*Develop. Biol.* 19, 498–526 (1969)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Walne, Patricia L., and A. H. Haber	Actions of acute gamma radiation on excised wheat leaf tissue. II. Immediate lethal dose effects on chloroplast ultrastructure and subsequent retardation of normal disintegration in darkness	*Radiation Botany* 8, 399–406 (1968)
Waters, L. C.	Altered chromatographic properties of tRNA from chloramphenicol-treated *Escherichia coli*	*Biochem. Biophys. Res. Commun.* 37, 296–304 (1969)
Wei, C. H.	Structural analyses of tetracobalt dodecacarbonyl and tetrarhodium dodecacarbonyl: Crystallographic treatments of a disordered structure and a twinned composite	*Inorg. Chem.* 8, 2384–97 (1969)
Wei, C. H., and L. F. Dahl	Triiron dodecacarbonyl: An analysis of its stereochemistry	*J. Am. Chem. Soc.* 91, 1351–61 (1969)
Weisberg, R. A., and M. E. Gottesman	The integration and excision defect of bacteriophage λ *dg*	*J. Mol. Biol.* 46, 565–80 (1969)
Wicks, W. D.	Induction of hepatic enzymes by adenosine 3′,5′-monophosphate in organ culture	*J. Biol. Chem.* 244, 3941–50 (1969)
	The possible role of cyclic adenylic acid in regulation of enzyme synthesis	Pp. 143–55 in *Regulatory Mechanisms for Protein Synthesis in Mammalian Cells*, ed. by Anthony San Pietro, M. R. Lamborg, and F. T. Kenney, Academic, New York and London, 1968
Wicks, W. D., F. T. Kenney, and Kai-Lin Lee	Induction of hepatic enzyme synthesis *in vivo* by adenosine-3′,5′-monophosphate	*J. Biol. Chem.* 244, 6008–13 (1969)
Williams, D. B.	Effects of radiation, antibiotics, and alkylating agents on cell division and growth in the ciliate *Spathidium spathula*	*J. Protozool.* 15, 811–16 (1968)
Williamson, J. H.	Identification of Y fragments with two doses of y^+ or B^S	*Drosophila Info. Serv.* 43, 157 (1968)
	On the nature of *Y* chromosome fragments induced in *Drosophila melanogaster* females. I. Immature oocytes	*Mutation Res.* 8, 327–35 (1969)
Williamson, J. H., and D. R. Parker	Recovery of multiple-break rearrangements from irradiated oocytes	*Drosophila Info. Serv.* 43, 178 (1968)
Yamada, Tuneo	Hans Spemann's contributions and contemporary biology	*Shizen* 12, 40–43 (1969)
Yamada, Tuneo, and Marion E. Roesel	Activation of DNA replication in the iris epithelium by lens removal	*J. Exptl. Zool.* 171, 425–31 (1969)

Lecture Programs

VISITING LECTURER PROGRAM

During 1969, 80 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 under the Visiting Lecturer Program. Twenty-one states and fourteen foreign countries – Australia, Belgium, Brazil, Czechoslovakia, England, France, India, Israel, Italy, Japan, The Netherlands, Scotland, Sweden, and Switzerland – were represented by the speakers. In addition to the guest lecturers listed below, there were numerous informal seminars at which Division members spoke about their own research. *An asterisk (*) indicates speakers who were cosponsored by the Biology Division and the University of Tennessee–Oak Ridge Graduate School of Biomedical Sciences under this program; a dagger (†) indicates sponsorship by the school alone.*

SPEAKER	DATE	AFFILIATION	SUBJECT
Mark Achtman	7/2/69	Department of Molecular Biology University of California Berkeley, California	A genetical analysis of sexual transfer in *E. coli*
Elinor Adman	10/29/69	Department of Biological Structure University of Washington Seattle, Washington	Structures of *cis-syn* uracil photodimer and its sulfur analog
T. V. Anadhani	5/9/69	Andhra University Waltair, India	Stochastic models in biology: A model of response after infection
R. E. Anderson	6/9/69	Department of Pathology University of New Mexico School of Medicine Albuquerque, New Mexico	The delayed consequences of ionizing radiation in atomic bomb survivors
G. A. Andrews	7/15/69	Medical Division ORAU Oak Ridge, Tennessee	Clinical research at ORAU Medical Division
K. C. Atwood	4/7/69	Department of Microbiology University of Illinois Urbana, Illinois	The genetic map location of rDNA in *Escherichia coli*
†Robert Auerbach	7/28/69	Department of Zoology University of Wisconsin Madison, Wisconsin	Developmental theory of immunity
T. G. Baker	5/12/69	Department of Obstetrics and Gynecology University of Edinburgh Edinburgh, Scotland	Factors affecting the radiosensitivity of mammalian oocytes with special reference to the human
*William Belser	5/2/69	University of California San Diego, California	Bacteriophage-episome interaction in *E. coli*
Robert Bernlohr	5/9/69	Department of Microbiology University of Minnesota Minneapolis, Minnesota	Enzyme inactivation during sporulation
Cesare Biancifiori	6/4/69	Istituto di Anatomia e Istologia Pathologica Divisione di Richerche sul Cancro Perugia, Italy	Some aspects of pulmonary tumorigenesis by hydrazine

SPEAKER	DATE	AFFILIATION	SUBJECT
†D. C. Birdsell	2/4/69	Department of Microbiology Scripps Clinic and Research Foundation La Jolla, California	Recent studies on the synthesis of *Bacillus subtilis* surface structures
Fred Blattner	7/2/69	McArdle Laboratory for Cancer Research University of Wisconsin Medical Center Madison, Wisconsin	The interaction of RNA polymerase with T7 DNA molecules
Ricardo Brentani	5/8/69	Department of Bioquimica Universidade de São Paulo São Paulo, Brazil	Characterization and metabolism of acyl dihydroxyacetone phosphate
A. Brito da Cunha	11/3/69	Departamento de Biologia Geral Universidade de São Paulo São Paulo, Brazil	Chromosomal activities and differentiation in *Sciaridae*
Michael Chen	5/15/69	University of California Berkeley, California	Studies on the proliferative capacity of macrophage progenitor cells
Clarence Colby	5/6/69	University of California Berkeley, California	Double stranded RNA in cells infected with a DNA virus
B. V. Conger	3/14/69	UT-AEC Oak Ridge, Tennessee	Modification of radiation damage in barley seeds
T. Timothy Crocker	10/7/69	Cancer Research Institute University of California School of Medicine San Francisco, California	Blocking of benzo(*a*)pyrene action in organ culture and in microsomal suspensions
S. R. Dickman	3/28/69	Department of Biochemistry University of Utah Salt Lake City, Utah	Purification and isolation of active mammalian ribosomal subunits
Jaroslav Drobnik	11/10/69	Charles University Prague, Czechoslovakia	The effect of ^{32}P decay on integrity of T7 DNA
Darrell Fleischman	12/5/69	C. F. Kettering Laboratory Yellow Springs, Ohio	Delayed fluorescence and membrane potentials in photosynthetic bacteria
Ernst Freese	4/21/69	Laboratory of Molecular Biology National Institute of Neurological Diseases and Blindness Bethesda, Maryland	Sporogenesis in *B. subtilis* as a model of differentiation
*F. H. Gaertner	3/18/69	Department of Biology University of California San Diego, California	Erythrose-4-phosphate and phosphenolpyruvate to indole-3-glycerol phosphate: Channeling *in vitro* by two multienzyme complexes in *Neurospora crassa*
R. C. Gallo	2/26/69	National Cancer Institute NIH Bethesda, Maryland	Changes in tRNA and tRNA methylation in human normal and neoplastic tissues
Mario Gosalev	9/17/69	Department of Biochemistry Fels Research Institute Temple University Philadelphia, Pennsylvania	Applications of differential spectrophotometry to cancer research: Recent findings in liver carcinogenesis
W. W. Harris	11/24/69	MAN Program Oak Ridge National Laboratory	Applications of the scanning electron microscope to biological problems
Robert Haselkorn	4/28/69	Department of Biophysics University of Chicago Chicago, Illinois	Regulation of gene expression in T4-infected bacteria
Walter E. Hill	4/8/69	Department of Biochemistry and Biophysics Oregon State University Corvallis, Oregon	Physical studies of ribosomal subunits from *E. coli:* Low angle x-ray scattering sedimentation equilibrium

SPEAKER	DATE	AFFILIATION	SUBJECT
C. F. Hollander	8/14/69	Experimental Gerontological Unit Organization for Health Research T.N.O. Rijswijk, The Netherlands	Syngeneic transplantation of the kidney of old rats into young recipients: Function, life-span, and histology
R. W. Holton	7/18/69	Department of Botany University of Tennessee Knoxville, Tennessee	Blue-green algae, protocells, and comparative biochemistry
Keiichi Hosokawa	1/17/69	Space Sciences Laboratory University of California Berkeley, California	The nature of binding of 5S RNA to 50S ribosomal subunit
John J. Hutton	10/13/69	Roche Institute of Molecular Biology Nutley, New Jersey	Genetic analyses of biochemical variants in mice
Prof. T. Iwamura	12/19/69	Faculty of Agriculture Nagoya University Chikusa, Nagoya, Japan	DNA species in algae
Bertil Järplid	4/23/69	Research Institute of National Defense Sundbyberg, Sweden	Radiation induced changes of thymus in mice
N. K. Jerne	11/5/69	Basel Institute of Immunology Basel, Switzerland	Biological mechanisms of aging
W. K. Joklik	10/16/69	Chairman, Department of Microbiology Duke University Durham, North Carolina	The molecular biology of reovirus
H. Kanatani	10/2/69	Bodega Marine Laboratory University of California, Berkeley Bodega Bay, California	Meiosis-inducing substance and its control: A study of spawning and oocyte maturation in starfish
L. J. Kaplan	3/13/69	Purdue University Lafayette, Indiana	The microheterogeneity of plasma albumin
Sohei Kondo	9/8/69	Professor of Fundamental Radiology Faculty of Medicine Osaka University Osaka, Japan	Photoreactivation kinetics in phage T1 and *E. coli* B_{s-1}: Evidence for localization of the photoreactivating enzyme
Gerhard Krüger	4/9/69	Laboratory of Pathology NCI Bethesda, Maryland	Histology of the host reaction against a tumor of known isoantigenicity
Katja Lakatos	6/16/69	Department of Physics and Astronomy University of Rochester Rochester, New York	The effect of lattice vibrations on trap-limited exciton lifetimes
Yehuba Lapidot	5/16/69	Department of Biological Chemistry Hebrew University Jerusalem, Israel	The chemical synthesis and some biological properties of oligopeptidyl-tRNA
K. S. Lavappa	1/13/69	Childrens Cancer and Research Foundation Harvard University Cambridge, Massachusetts	Meiosis in the male Armenian hamster
L. Ledoux	6/23/69	Department of Radiobiology Laboratoires du C. E. N. Mol-Donk, Belgium	New perspective on the uptake of the information in DNA by living tissues
N. J. Leonard	9/26/69	Department of Chemistry and Chemical Engineering University of Illinois Urbana, Illinois	Synthetic spectroscopic models related to coenzymes and base pairs
Patricia J. Lindop	5/13/69	The Medical College of St. Bartholomew's Hospital London, England	Interpretation of dose response curves for radiation-induced tumors

SPEAKER	DATE	AFFILIATION	SUBJECT
A. W. Linnane	8/25/69	Department of Biochemistry Monash University Clayton, Victoria, Australia	The nature of mitochondrial protein synthesis and some aspects of its genetic control
F. M. Lovell	3/17/69	Department of Biochemistry Columbia College of Physicians and Surgeons New York, New York	Some approaches to the structure of insulin and oxytocin
J. R. Maisin	11/3/69	Laboratoires du C.E.N. a Mol-Donk Bruxelles, Belgium	Influence of radioprotectors on radiation induced pneumonitis, malignant diseases, and subsequent longevity of mice
Shraga Makover	3/28/69	Carnegie Institute of Washington Genetics Research Unit Cold Spring Harbor, New York	The origin and direction of replication of lambda bacteriophage DNA
R. E. McCarty	5/23/69	Biochemistry and Molecular Biology Section Cornell University Ithaca, New York	The uncoupling of photosynthetic phosphorylation by ammonium salts
†Elias Meezan	2/18/69	Department of Biology California Institute of Technology Pasadena, California	Comparative studies on the membrane glycoproteins of normal and virus-transformed mouse fibroblasts
T. J. Mitchell	12/18/69	Statistics Group Mathematics Division Oak Ridge National Laboratory	Statistical analysis on the time share computer
Sankar Mitra	9/5/69	Bose Institute Calcutta, India	Alterations in the surface properties of *E. coli* after infection with bacteriophage M13
M. A. S. Moore	11/20/69	The Walter and Eliza Hall Institute of Medical Research Royal Melbourne Hospital Victoria, Australia	Development and senescence of the hemopoietic system
Gisela Mosig	6/24/69	Department of Molecular Biology Vanderbilt University Nashville, Tennessee	The control of T4 DNA replication
Michio Oishi	10/14/69	Public Health Laboratory of the City of New York New York, New York	Purification and characterization of an ATP dependent DNAase from *E. coli*
Ingram Olkin	4/11/69	Stanford University Stanford, California	A multivariate test of the equivalence of two drugs
Lee Pratt	11/14/69	Department of General Biology Vanderbilt University Nashville, Tennessee	Pathways of phytochrome phototransformation
R. J. Preston	11/21/69	Radiological Research Unit Harwell, Didcot, Berkshire, England	Chromosome aberrations and cell-killing at different radiation qualities
Arnold Ravin	10/20/69	Department of Biology University of Chicago Chicago, Illinois	Integration of DNA during bacterial transformation: Influence of the donor marker and its neighborhood
Umberto Saffiotti	3/19/69	National Cancer Institute Bethesda, Maryland	Lung carcinogenesis
†Thomas Schleich	3/10/69	Department of Chemistry and Institute of Molecular Biology University of Oregon Eugene, Oregon	Specific ion effects on the solution conformation of poly-L-proline

SPEAKER	DATE	AFFILIATION	SUBJECT
*David Schlessinger	10/2/69	Department of Microbiology School of Medicine Washington University St. Louis, Missouri	Messenger RNA metabolism in *E. coli*
O. J. Schwarz	6/9/69	Department of Botany North Carolina State University Raleigh, North Carolina	Thymidine kinase in plants
June R. Scott	6/23/69	The Rockefeller University New York, New York	Lysogeny in phage P1
Peter Setlow	12/22/69	Department of Biochemistry Stanford University Stanford, California	Biochemical studies of bacterial sporulation and germination
Michel Sicard	6/16/69	Laboratoire de Genetique Faculté des Sciences Université de Toulouse Toulouse, France	Mode of integration of high and low efficiency markers in transformation of pneumococcus
E. R. Signer	4/10/69	Department of Biology Massachusetts Institute of Technology Cambridge, Massachusetts	Recombination in lambda
Howard E. Skipper	12/3/69	Kettering-Meyer Laboratories Southern Research Institution Birmingham, Alabama	Experimental therapeutics in the cancer area is becoming a more rational business
J. F. Spalding	1/21/69	Los Alamos Scientific Laboratory Los Alamos, New Mexico	A within-strain difference in radiation sensitivity in the RFM mouse
*Russell Steere	11/12/69	Plant Virology Laboratory Crops Research Division Agricultural Research Service U.S. Department of Agriculture Beltsville, Maryland	New approaches in freeze-etch studies of inner and outer surfaces of membranes
Himan Sternlicht	4/29/69	Department of Chemistry University of California Berkeley, California	^{13}C NMR of amino acids and polypeptides
Tian Yow Tsong	2/7/69	Yale University New Haven, Connecticut	Optical activities of cytochrome b_2
G. Venema	6/26/69	Genetisch Instituut Rijksuniversiteit Te Groningen Haren, The Netherlands	*Haemophilus influenzae* transformation and rec$^-$ strains
Jean-Pierre Waller	6/19/69	Centre National de la Recherche Scientifique Laboratoire d'Enzymologie Gif-sur-Yvette, France	The amino acylation of *E. coli* valine tRNA by yeast phenylalanyl-tRNA synthetase
Luther S. Williams	9/19/69	Department of Biology Atlanta University Atlanta, Georgia	Role of valine transfer RNA in the regulation of RNA and biosynthetic enzyme synthesis in *E. coli*
Randall Wood	10/1/69	Medical Division Oak Ridge Associated Universities Oak Ridge, Tennessee	Analysis and metabolism of lipids of Ehrlich ascites cells
James Zissler	6/20/69	Department of Biology Massachusetts Institute of Technology Cambridge, Massachusetts	Mutants of phage λ unable to grow in *rec* bacteria

UNIVERSITY OF TENNESSEE–OAK RIDGE GRADUATE SCHOOL OF BIOMEDICAL SCIENCES–BIOLOGY DIVISION DISTINGUISHED LECTURER SERIES

During 1969 the following visiting scientists presented lectures under the Distinguished Lecturer series begun in 1967 under the joint sponsorship of the University of Tennessee–Oak Ridge Graduate School of Biomedical Sciences and the Biology Division:

SPEAKER	DATE	AFFILIATION	SUBJECT
B. W. Agranoff	12/4/69	Department of Biochemistry and Mental Health Research Institute University of Michigan Ann Arbor, Michigan	Antimetabolites and memory formation
D. B. Amos	2/13/69	Department of Microbiology and Immunology Duke University Medical Center Durham, North Carolina	Human leukocyte and tissue antigens
Paul Berg	5/22/69	Department of Biochemistry Stanford University Stanford, California	Induction of viral multiplication in polyoma transformed cells
L. Luigi Cavalli-Sforza	9/8/69	Department of Genetics Stanford University School of Medicine Stanford University Medical Center Stanford, California	Quantitative study in human evolution
L. E. Orgel	3/21/69	The Salk Institute of Biological Studies San Diego, California	Template activity without enzymes
T. T. Puck	6/27/69	Institute for Cancer Research University of Colorado Medical Center Denver, Colorado	Human cell genetics
R. T. Schimke	10/30/69	Department of Pharmacology Stanford University School of Medicine Stanford, California	Mutations in inbred mouse strains and the study of regulatory mechanisms in mammals
E. Donnell Thomas	11/14/69	Division of Oncology University of Washington Seattle, Washington	Problems and progress in marrow transplantation in outbred species
P. H. von Hippel	3/27/69	Institute of Molecular Biology University of Oregon Eugene, Oregon	Specific ion effects on the solution structure of biological macromolecules

TRAINING IN AGING LECTURES

Under the sponsorship of the University of Tennessee–Oak Ridge Graduate School of Biomedical Sciences, through a grant from the National Institute of Child Health and Human Development, a new series of special lectures oriented toward the Training in Aging program was begun:

SPEAKER	DATE	AFFILIATION	SUBJECT
Elizabeth D. Hay	12/19/69	Department of Anatomy Harvard Medical School Cambridge, Massachusetts	The problem of dedifferentiation and cell metaplasia in regeneration
F. S. Sinex	10/24/69	Chairman, Department of Biochemistry Boston University School of Medicine Boston, Massachusetts	The role of the nucleus in aging

Meetings and Conferences in 1969

ANNUAL RESEARCH CONFERENCE

The twenty-second annual Biology Division Research Conference was held in Gatlinburg, Tennessee, March 31–April 3. Titled **Symposium on Protein–Nucleic Acid Interaction,** the conference included papers on the basic structure of nucleic acids and proteins, the association of nucleic acid enzymes with their specific substrates, the biological modification of nucleic acids (especially transfer RNA), and the structure of macromolecules such as ribosomes and bacteriophages.

The organizing committee was headed by Elliot Volkin and included W. E. Cohn, K. Bruce Jacobson, J. W. Longworth, Peter Pfuderer, and R. B. Setlow.

Published as a supplement to the October issue of the *Journal of Cellular Physiology,* the proceedings include two papers by Division investigators: "The separation of isoaccepting transfer RNA's and the possible role of tRNA in regulation" by G. David Novelli and "Studies of DNA-dependent RNA polymerase" by Audrey Stevens. Following the latter paper, O. L. Miller, Jr., discussed at length his electron micrographs of nucleic acid in action; his remarks are given in the proceedings as a discussion paper, "Portrait of a gene," with Barbara R. Beatty as coauthor.

AEC-NIGMS CHEMICAL MUTAGENESIS REVIEW

Two representatives of the National Institute of General Medical Sciences and an *ad hoc* committee visited the Biology Division June 4 to review the AEC-NIGMS Cooperative Program on chemical mutagenesis. General objectives as well as past, present, and future work were discussed by W. L. Russell, W. M. Generoso, and R. B. Cumming in a seminar on research sponsored by this program in the Division. Afterward, the visitors conferred individually with investigators. Abstract booklets excerpted from the 1968 Biology Division Annual Progress Report were available to the visitors.

Participating in the review were C. W. Edington, Division of Biology and Medicine, U.S. Atomic Energy Commission; George W. Woolley and Arthur E. Heming, National Institute of General Medical Sciences; Seymour Abrahamson, University of Wisconsin; Gerald P. Fink, Cornell University; Alfred G. Knudson, Jr., State University of New York at Stony Brook; and Edward E. Lewis, California Institute of Technology.

AEC-NCI CARCINOGENESIS REVIEW

Visiting the Biology Division June 5–6 to review the AEC-NCI Carcinogenesis Program were 16 scientists from the U.S. Atomic Energy Commission and the National Cancer Institute.

On June 5 the visiting scientists heard reports of research in the Division, and on June 6 they had an opportunity to confer with individual investigators. Abstracts covering the cooperative program on carcinogenesis were excerpted from the 1968 Biology Division Annual Progress Report for the visitors.

Representing NCI were C. G. Baker, R. R. Bates, C. W. Boone, Earle Browning, M. S. Fish, Gio Gori, John Hartinger, G. E. Meyer, B. H. Morrison, J. A. Peters, Umberto Saffiotti, and Jesse Steinfield. From the Division of Biology and Medicine, USAEC, were C. W. Edington and J. B. Storer. H. P. Rusch, from the McArdle Laboratory for Cancer Research, Madison, Wisconsin, also attended the review.

SYMPOSIUM ON GNOTOBIOTIC RESEARCH

The Association for Gnotobiotics convened in Oak Ridge June 10–13 for its eighth annual meeting – a symposium on **Gnotobiotic Research: Its Importance to Basic Mammalain Research and Human Medicine.**

The primary aim of the symposium, as set forth at the beginning of the conference by H. E. Walburg, Jr., the symposium chairman, was critical analysis of current directions in gnotobiotic research. Sessions were held on various physiological effects in germfree and conventional animals, microbial flora and its influence on immunological mechanisms, radiation effects in germfree and conventional mice, immunology and infectious mechanisms, and gnotobiotic technology.

Two papers from the Biology Division were presented: "Genetics of bacteria *in vivo* as related to microbial ecology" by R. T. Jones, Roy Curtiss III, and H. E. Walburg, Jr., and "Cardiovascular and renal lesions in unirradiated germfree and conventional mice in a life-span study" by G. E. Cosgrove and H. E. Walburg, Jr.

The symposium was sponsored by Oak Ridge National Laboratory and the Association for Gnotobiotics.

FOURTH CONFERENCE ON BLOOD PLATELETS

The fourth **Conference on Blood Platelets** was held in Oak Ridge June 23–24. Sponsored by Oak Ridge National Laboratory, the conference included over 50 reports of research on various aspects of the field: carbohydrates, fats, and proteins of platelets; platelet and megakaryocyte kinetics; hemostasis; the release reaction and inhibitors of release; platelet aggregation; and platelet diseases, membranes, and preservation. Several papers were by foreign investigators. One paper from the Biology Division was presented: "Ploidy composition of megakaryocyte stages" by T. T. Odell, Jr., and C. W. Jackson.

Previous conferences in the series were held in 1962, 1964, and 1967. T. T. Odell, Jr., has organized these meetings.

CHEMICAL PROTECTION AGAINST IONIZING RADIATION

Several investigators from the Biology Division met with a group of about 20 scientists invited by the Division of Biology and Medicine (U.S. Atomic Energy Commission) to participate in a working conference on **Chemical Protection Against Ionizing Radiation.**

Hosted by the Biology Division in Gatlinburg, Tennessee, July 9–11, this was the third meeting in a series of colloquia held twice yearly under AEC sponsorship in various parts of the country. The purpose of the series is to keep the Division of Biology and Medicine abreast of developments and problems in chemical protection. With an informal agenda, the attendees survey and discuss these problems and evaluate the direction of current research in the field.

C. C Congdon, D. G. Doherty, L. H. Smith, and J. B. Storer represented the Biology Division at the conference.

CONFERENCE ON INHALATION CARCINOGENESIS

The Biology Division was host to some 150 investigators and representatives of government and industry at the **Conference on Inhalation Carcinogenesis**, sponsored by the National Cancer Institute and the U.S. Atomic Energy Commission and held October 8–11 in Gatlinburg, Tennessee.

The program began with a symposium on the relation of inhalation exposure to carcinogenesis, chaired by J. L. Liverman, an Assistant Director of Oak Ridge National Laboratory. This symposium was followed by sessions on inhalation technology, cellular and functional injury following inhalation exposure, respiratory carcinogenesis, current studies on inhalation carcinogenesis, and program planning in the field by NCI, AEC, and NIEHS. Paul Nettesheim of the Biology Division presented a paper, "Effects of chronic exposure to artificial smog and chromium oxide dust on the incidence of lung tumors in mice," coauthored by M. G. Hanna, Jr., D. G. Doherty, R. F. Newell, and Alfred Hellman.

On the organizing committee were M. G. Hanna, Jr. (chairman), Paul Nettesheim, C. R. Richmond, and Umberto Saffiotti. Proceedings of the conference will be published by the Division of Technical Information Extension, U.S. Atomic Energy Commission, Oak Ridge.

ANNUAL INFORMATION MEETING

The annual visit of the Oak Ridge National Laboratory Advisory Committee for Biology took place November 17–19. After Division investigators summarized their work for the group at the **Annual Information Meeting,**

members of the committee visited the various laboratories and participated in informal discussions. A booklet of abstracts covering all aspects of research in the Division was prepared for their use.

Committee members attending were Rollin D. Hotchkiss, Rockefeller University; Henry S. Kaplan, Stanford University Medical School; C. L. Markert, Yale University; R. D. Owen, California Institute of Technology; and Herschel Roman, University of Washington.

INTERNATIONAL SYMPOSIUM ON THE FERTILITY OF THE SEA

The ninth in the continuing series of Latin-America conferences initiated in 1961 under the leadership of Alexander Hollaender was held in São Paulo, Brazil, December 1–6. The conference, **Symposium on the Fertility of the Sea,** was concerned with both practical and academic problems. Physical, chemical, and geological oceanographic processes relating to the enrichment and fertility of the sea were assessed, and ecological distribution of marine organisms and biological consequences of processes that enrich the marine environment were evaluated. Special attention was given to features of the oceans around Latin America.

The following organizations cooperated in sponsoring, planning, and supporting the conference: The Oceanographic Institute of the University of São Paulo, with the support of the São Paulo State Research Foundation and the Brazilian National Research Council; the Ford Foundation, via a grant administered by the U.S. National Academy of Sciences; the U.S. Atomic Energy Commission and the National Science Foundation through grants to Duke University; Duke University; and the Biology Division of the Oak Ridge National Laboratory.

Serving on the organizing committee for the meeting were M. Vannucci (chairman), Oceanographic Institute of the University of São Paulo; Alexander Hollaender (cochairman), Biology Division, Oak Ridge National Laboratory; L. R. A. Capurro, Texas A&M University and the National Institute of Oceanography, Argentina; L. B. Cavalcanti, Laboratory for Sciences of the Sea, Federal University of Pernambuco, Brazil; J. D. Costlow, Jr., Duke University Marine Laboratory; A. M. Couceiro, National Research Council, Brazil; M. da Frota Moreira, National Research Council, Brazil; H. Jakobi, Department of Zoology, Federal University of Paraná, Brazil; P. C. Moreira da Silva, Foundation for Studies of the Sea, Brazil; M. Pinto Paiva, Marine Biology Station, Federal University of Ceará, Brazil; T. J. Smayda, Narragansett Marine Laboratory, University of Rhode Island; J. D. H. Strickland, University of California; J. G. Tundisi, Oceanographic Institute of the University of São Paulo; K. M. Wilbur, Duke University; and W. S. Wooster, Scripps Institution of Oceanography.

BONE MARROW CONFERENCES

Since 1957, the Biology Division has cooperated with investigators from many institutions throughout the world in arranging at least two conferences each year on bone marrow transplantation and chemical protection. In most years since that time, a third (occasionally a fourth) conference on related specific topics has been convened. Special emphasis is placed on developing human applications from animal experiments. In 1969, only two conferences were held.

Abstracts of the conference reports, invited papers on selected subjects pertinent to a particular meeting, and contributed papers in the field of hematology are published in *Experimental Hematology,* a journal edited in the Biology Division by C. C Congdon and the editorial office and published by the ORNL Technical Information Division.

An evening bone marrow session was held April 14 at Atlantic City, New Jersey, in conjunction with the meetings of the Federation of American Societies for Experimental Biology. The bone marrow session was organized and chaired by J. P. OKunewick of Allegheny General Hospital. The proceedings were published in *Experimental Hematology,* No. 19, along with four contributed papers.

The winter bone marrow conference, held December 4–5 in Baltimore, Maryland, was organized by George Santos of Johns Hopkins University. Special emphasis fell on the graft-vs-host syndrome, its mechanisms, prevention, and treatment. In addition to the clinical, pathological, physiological, and functional consequences of graft-vs-host disease, its therapeutic uses were discussed and evaluated. *Experimental Hematology,* No. 20, contains abstracts from this meeting, as well as eight contributed papers.

BIOMEDICAL DIRECTORS MEETINGS

Representatives from the Biology Division attended three meetings of the Biomedical Program Directors of the U.S. Atomic Energy Commission this year. These meetings are held periodically for review of research in the various programs supported by the AEC through its Division of Biology and Medicine.

The directors convened February 10–11 in Salt Lake City, Utah; May 12–13 in Boston, Massachusetts; and October 13–14 in Bethesda, Maryland.

Educational Activities

STUDENT TRAINEE PROGRAM

This year 15 juniors from colleges in 11 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 the Division staff members.

The 1969 students, their affiliations, and the groups to which they were assigned are:

Name	School	Group
Louvenia D. Carter	Stillman College Tuscaloosa, Alabama	Pathology and Immunology
Kathryn Y. Du	William Smith College Geneva, New York	Pathology and Immunology
Shirley A. Ellis	Miles College Birmingham, Alabama	Mammalian Recovery
Sandra G. Feese	Marshall University Huntington, West Virginia	Mammalian Cytology and Cell Genetics
Saramarie L. Hackette	University of California Davis, California	Biophysics
Kathryn J. Kull	Muhlenberg College Allentown, Pennsylvania	Cell Growth and Differentiation
Mini A. Liu	Radcliffe College Cambridge, Massachusetts	Molecular Photobiology of DNA
Michael J. Montague	University of Michigan Flint College Flint, Michigan	Enzymology
Patricia J. Pate	Siena Heights College Adrian, Michigan	Plant Physiology and Photosynthesis
Mary A. Peters	Sacred Heart College Wichita, Kansas	Enzymology
Elliott M. Ross	University of California Davis, California	Cell Physiology
Richard L. Siegel	Washington University St. Louis, Missouri	Radiation Microbiology and Microbial Genetics
Emily J. Turley	Randolph-Macon Woman's College Lynchburg, Virginia	Mammalian Genetics
Kenneth H. Walbert	Pennsylvania State University University Park, Pennsylvania	Biophysics
William B. Ware	Colby College Waterville, Maine	Enzymology

STUDENT EMPLOYEES

There were 13 undergraduate students who worked in the Division on temporary summer appointments. Listed below are their names, the colleges they attend, and the groups with which they were associated:

Name	School	Group
Janice L. Altum	East Tennessee State University Johnson City, Tennessee	Administrative Office
Rebera E. Elliott	Fisk University Nashville, Tennessee	Enzymology
Bonnie K. Ferrill	Tennessee Technological University Cookeville, Tennessee	Administrative Office
Jessie V. Furlow	Lane College Jackson, Tennessee	Enzymology
Iris L. McMillin	The University of Tennessee Knoxville, Tennessee	Administrative Office
Sharon K. Murphy	The University of Tennessee Knoxville, Tennessee	Administrative Office
Bettie L. Nelson	Virginia State College Petersburg, Virginia	Cell Growth and Differentiation
Katherine E. Settles	Knoxville College Knoxville, Tennessee	Biophysics; Cell Physiology

In addition, the following temporary employees worked in the Division during the summer:

Name	Group
Nancy E. Moulden	Fungal Genetics Group
Mary J. Powell	Nucleic Acid Chemistry
James L. Short	Nucleic Acid Enzymology
Phillip N. Turman	Administrative Office
Leroy C. Turner	Administrative Office

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

The third academic year of the Graduate School of Biomedical Sciences began in September 1969. The 28 students enrolled are from 18 states, Panama, and Canada, and another student was accepted for January admission from Chile. Eight students are engaged in doctoral research, and one (J. C. Bagshaw) received his Ph.D. in December and accepted an appointment at Harvard Medical School – Massachusetts General Hospital.

During 1969, Colonel Robert L. Breeding was appointed Assistant to the Director to aid Director R. C. Fuller in the administrative development of the school; Dr. Frank H. Gaertner, previously Assistant Professor at the University of California, San Diego, was appointed Assistant Professor in the school; Dr. D. E. Olins, formerly an Assistant Professor, became an Associate Professor; and Professor John S. Cook continued as Associate Director to assist the Director in the academic development of the school. In addition to this full-time University staff, 69 members of the Oak Ridge National Laboratory scientific staff (67 from the Biology Division) were engaged in teaching or supervising student research on a part-time basis. Dr. Robert R. Becker, Professor in the Department of Biochemistry and Biophysics at Oregon State University, taught a course in protein chemistry during a four-week appointment as Visiting Professor in the school during July and August 1969.

In addition to the five-year training grant of $445,500 from the National Institute of General Medical Sciences (NIH), awarded in 1968, the school received a five-year grant of $496,656 for a training program in Aging Research from the National Institute of Child Health and Human Development (NIH) and a one-year Postdoctoral Investigatorship Award of $90,000 from Union Carbide Corporation. Dr. Olins is supported by an NIH Career Development Award, and the following students were awarded national competitive fellowships: Ann E. Campbell, Stephen A. Fairfield, Sarah A. Goodman, and Dale E. Graham – AEC-ORAU Predoctoral Fellowships; Gary R. Dunn, Gerald B. Price, and Kenneth J. Roozen – NIH Predoctoral Fellowships; and Paul B. Selby – NSF Predoctoral Fellowship.

Three students are supported by NDEA Title IV Fellowships, 2 by appointments as Graduate Research Assistants, and 13 by the NIGMS Training Grant; the student from Chile will be supported by the Organization of American States.

Eight students are engaged in thesis research, and the first Ph.D. was awarded to Joseph C. Bagshaw in December, 1969.

In addition, there are 10 postdoctoral trainees appointed to the Oak Ridge National Laboratory Biology Division and supported by grants awarded to the school – four by the Aging Research Grant and six by the Postdoctoral Investigatorship Grant.

The school continues to operate in its temporary quarters within the Biology Division while plans are being completed for construction of its own facility in 1971. The school's Student Center, located in the University apartment housing area, has been completed and furnished and is being used extensively for informal sessions on science between visiting scientists and students.

As part of the school's continuing effort to recruit qualified students, staff members visited a number of campuses throughout the United States during the year. Additional visits are planned in 1970.

In cooperation with the Biology Division, the Graduate School of Biomedical Sciences continued to cosponsor the Distinguished Lecturer Series, which brings distinguished speakers to the laboratory under the joint auspices of both institutions. Lecturers under this program, and under the Training in Aging Program, which is sponsored by the school only (through a grant from the National Institute of Child Health and Human Development) are listed on p. 21.

In addition, the school sponsored a number of speakers on the Visiting Lecturer Program, several in cooperation with the Biology Division and several under sole sponsorship [cosponsored lecturers are indicated by an asterisk (*) and lecturers sponsored by the school alone are indicated by a dagger (†) on the list of visiting lecturers, pp. 16–20].

COURSES TAUGHT

UT–Oak Ridge Resident Graduate Program – The Oak Ridge Resident Graduate Program, sponsored jointly by the University of Tennessee and Oak Ridge Associated Universities, Inc. (ORAU), has been an integral part of the UT Graduate School since 1945. This program offers graduate instruction in science and engineering and is designed primarily to further the professional advancement of employees of the three U.S. Atomic Energy Commission installations located in Oak Ridge. Classes are held at the ORAU Special Training Building in Oak Ridge.

Several members of the Biology Division have taught courses under this program during the past year. Brief descriptions of the courses, and names of those teaching, follow:

Advanced Human Genetics covered, in particular, human mutations as exhibited by human mutant cells *in vitro*. James D. Regan

General Genetics concerned fundamental principles of genetics, including the chemical basis of heredity, along with current concepts of mutation, gene structure, and gene function on a molecular level. J. L. Epler

Microtechnique involved three methods for microscopic study of animal tissues: preparation of paraffin sections treated with a variety of stains for identification of particular substances; autoradiographic localization of radioisotopes; and histochemical identification of substances, such as mucopolysaccharides, enzymes, and pigments. W. D. Gude

Molecular Genetics dealt with translation, transcription, regulation, mutation, suppression, transduction, and transformation. W. E. Barnett

UT–ORNL–Ford Foundation Cooperative Research Program – Through the UT-ORNL Cooperative Research Program, initiated by funds from the Ford Foundation, investigators from the Laboratory are appointed to the University faculty and serve on a part-time basis. Three members of the Biology Division participated in the program in 1969.

E. H. Y. Chu taught a series of advanced seminars in genetics during the winter and spring. The series deals with modern trends and theories in genetics.

C. C Congdon taught four courses in comparative animal pathology in the winter and spring quarters. Pathological processes in cells and tissues, and their causes, were covered in the winter; and changes in the organ, organ system, and organism were covered in the spring.

A. H. Haber taught an undergraduate course in fundamentals of botany during the winter. It concerned the nature and development of plants and included plant physiology, anatomy, morphology, life histories, and inheritance. In the spring, Dr. Haber taught a graduate course in developmental plant physiology. The growth and differentiation of higher plants were considered at molecular, cellular, and organismic levels of complexity; topics discussed in the course included chemical regulation of development, mathematical aspects of growth, interrelationships of cell division and expansion in development, macromolecular interpretations of differentiation, photoperiodism and endogenous rhythms, dormancy, and senescence.

Other courses – Two courses offered by the Department of Zoology at the University of Tennessee were taught by Biology Division personnel last year. As in recent years, James D. Regan taught the Human Genetics course in the spring. During the summer quarter, W. D. Gude taught the Histology course.

LECTURES GIVEN IN COURSES, INSTITUTES, AND SPECIAL PROGRAMS

Several members of the Biology Division gave lectures during the Summer Institute for College Science Teachers given by Oak Ridge Associated Universities during July and August. H. I. Adler spoke on genetic control of radiation sensitivity, W. D. Gude spoke on autoradiography and conducted two laboratory sessions, A. H. Haber lectured on radiobotany, and Ellen Mattingly spoke and conducted a laboratory session on high-resolution autoradiography in the study of insect physiology.

A graduate course in radiation physiology, offered last spring by the Department of Zoology at the University of Tennessee and directed by Darrell Q. Brown of the University, was taught almost entirely by Biology Division personnel. H. I. Adler, E. H. Y. Chu, and D. G. Doherty each presented two lectures; G. E. Cosgrove, E. F. Oakberg, R. O. Rahn, W. L. Russell, L. H. Smith, P. A. Swenson, and A. C. Upton each lectured once.

Three investigators from the Biology Division participated in teaching a course on Modern Topics in Biology at the Mayaguez campus of the University of Puerto Rico. H. I. Adler organized the course and taught in February; C. M. Steinberg taught in March and J. L. Epler in April.

L. G. Caro organized and taught a one-week advanced course on high-resolution and quantitative autoradiography at the Institute for Scientific Investigation, Caracas, Venezuela, in the late spring.

In March, E. H. Y. Chu lectured on evolutionary patterns in cytogenetics in a postdoctoral course on genetics and cytogenetics at the Institute for Medical Research in Camden, New Jersey.

On April 17, 1969, G. E. Cosgrove gave the sixth in a series of Public Awareness Seminars at the Regional Science Experience Center, a PACE (Projects to Advance Creativity in Education) center located in Oak Ridge. Dr. Cosgrove discussed the biology of disease and the similarities and differences in diseases among the various forms of life.

Roy Curtiss III spoke on bacterial conjugation to a microbial genetics class at Temple University (Philadelphia, Pennsylvania) in February, to a genetics class at Vanderbilt University (Nashville, Tennessee) in April, and to students taking a course on bacterial genetics at Cold Spring Harbor Laboratory (Cold Spring Harbor, New York) in July. He also lectured on bacterial pili for a course in bacterial physiology at the University of Tennessee in May.

Three members of the Biology Division spoke in an undergraduate course, Concepts in General Zoology, at the University of Tennessee last autumn. Roy Curtiss III gave the first lecture, "Definition and origin of life: An introduction to biological science." D. G. Doherty gave two lectures on the chemistry of living matter – covering nucleic acids, proteins, carbohydrates, and lipids. W. D. Gude discussed cellular organization of organisms, specialization of cells, kinds of tissues, and organ systems in two lectures on histology.

Rhoda F. Grell, O. L. Miller, Jr., and Liane B. Russell lectured to a graduate class in a course on cytogenetics taught by Sandra Bell at the University of Tennessee. In April, Dr. Miller spoke on the fine structure of genetic activity; in May, Dr. Russell discussed the mammalian X chromosome, and Dr. Grell talked on meiosis in *Drosophila melanogaster* females.

W. D. Gude lectured on autoradiography in three Basic Research Courses offered by Oak Ridge Associated Universities last year. The first course was conducted April 21–May 16; the second, June 16–July 11; and the third, September 8–October 3.

F. T. Kenney was in Baltimore March 10–11 to lecture in a graduate course in Biochemistry at the University of Maryland School of Medicine. As Merck, Sharp, and Dohme Visiting Professor of Biochemistry, he spoke on mechanisms of hormonal regulation and protein synthesis in a biochemistry course at the University of Nebraska School of Medicine (Omaha) in May. In December, Dr. Kenney gave two lectures on mechanisms of hormone action as a Claude Bernard Professor at the Institute of Experimental Surgery, University of Montreal.

As a Research Professor of Biochemistry in the cooperative graduate and postdoctoral teaching program between the University of Georgia and the Biology Division, G. David Novelli gave three lectures on protein synthesis in late winter and two lectures in late spring at the University of Georgia, Athens.

In September, two investigators from the Biology Division gave lectures at the NATO Advanced Study Institute on Photodynamic Action held at Sassari, Sardinia, Italy. R. B. Setlow spoke on the methodology of irradiation and the analysis of irradiated material, and Jane K. Setlow spoke on repair of biological systems by light.

In February, A. C. Upton presented a series of invited lectures as Visiting Professor of Pathology at the University of New Mexico School of Medicine in Albuquerque. Dr. Upton also spoke to undergraduate biology students and medical students at the University of Arkansas (Little Rock) as an AIBS guest lecturer in May.

Division Members Working in Other Laboratories

TO CONTINUE EDUCATION

M. L. Davis, who continued his studies and research for the Ph.D. degree in radiation biology at the University of Tennessee, received his degree in August. Dr. Davis has been on leave of absence from the Pathology and Physiology Unit, sponsored by a U.S. Public Health Service Predoctoral Fellowship. He is presently a staff member of the Molecular Anatomy Program, Oak Ridge National Laboratory.

R. T. Jones is on leave of absence from his position in the Pathology and Physiology Unit to continue work on a Ph.D. degree in bacteriology at the University of Tennessee. Mr. Jones is a U.S. Public Health Service Predoctoral Fellow, and his thesis research is being carried out in the Division's Radiation Microbiology and Microbial Genetics Group under the direction of Roy Curtiss III. He will complete his research in December 1969.

D. H. Reese is working on a Ph.D. degree in zoology at the University of Tennessee as a U.S. Public Health Service Predoctoral Fellow. Mr. Reese is on leave of absence from the Division's Cell Growth and Differentiation Group, and his thesis research is being carried out in the same group under the direction of Tuneo Yamada. He will complete his research in December 1969.

AT THE ATOMIC ENERGY COMMISSION

W. D. Fisher is spending a 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.

G. E. Stapleton, who has spent the past three years at the Division of Biology and Medicine of the Atomic Energy Commission, Washington, D.C., joined their permanent staff in June 1969. Dr. Stapleton has been on leave of absence from the Biology Division.

1. Biochemistry Section

Elliot Volkin

Chemical Protection and Enzyme Catalysis

D. G. Doherty
Jesse James[a]
J. R. Vercelloti[a]

Biochemistry of Cell Differentiation

John Papaconstantinou
W. S. Bradshaw[b]
Jean Delcour[b,c]
E. F. DuBrul[b]

Enzymology Unit – G. D. Novelli

Protein Synthesis

G. D. Novelli[d]
Audrey N. Best
A. A. Francis
J. M. Frazer[b]
A. P. Pfuderer
E. F. Phares
Lawrence Rosen
Helen G. Sellin
Audrey L. Stevens
L. C. Waters
C. J. Wust
Wen-Kuang Yang

Enzymatic Mechanisms in Protein Synthesis

M. P. Stulberg
J. G. Farrelly[b]
L. R. Shugart

Enzyme Regulation

K. B. Jacobson
J. E. Strickland[b]

Nucleic Acid Chemistry

W. E. Cohn
R. D. Faulkner[b]
B. C. Pal
Mayo Uziel

Nucleic Acid Enzymology

Elliot Volkin
W. E. Barnett
J. L. Epler
R. K. Fujimura
J. X. Khym
C. G. Mead
S. K. Niyogi
W. S. Riggsby

Developmental Biochemistry

F. J. Finamore
R. D. Ewing[b]
A. L. Golub[b]

[a]Consultant.
[b]Postdoctoral investigator.
[c]Visiting investigator from abroad.
[d]Leave of absence.

1.1 STRUCTURE AND FUNCTION OF $tRNA^{Phe}$

Mayo Uziel A. Jeannine Bandy
R. D. Faulkner Chongkun Koh

The *E. coli* B grown at the Oak Ridge National Laboratory contains at least two chromatographically distinct species of $tRNA^{Phe}$. These represent more than 90% of the total $tRNA^{Phe}$ extracted from the cells. Our previous reports have been concerned with $tRNA^{Phe}$ present in the more strongly retained peak. We have now prepared both $tRNA^{Phe}$ (I and II) in greater than 95% purity. The two preparations have virtually identical oligonucleotide maps, and, contrary to our previous report, the anticodon region consists of the sequence ψ-U-G-A-A-A*-A-ψ, where A* is 2-methylthio-N^6-isopentenyladenosine.

Since the large fragments used to prove the sequence of $tRNA^{Phe}$ from residues 1 to 30 had been obtained in relatively low yield, we repeated the borohydride reduction experiment at a lower pH, where random internucleotide hydrolysis is minimized. In addition to the improved oligonucleotide stability, the 4-thiouridine surprisingly was not reduced. The large fragments produced by RNase A hydrolysis are being characterized.

The reaction of mono- and bifunctional reagents with $tRNA^{Phe}$ has been used to probe the conformation of tRNA in solution. Formaldehyde, which is capable of methylol formation or of bridging two purine residues, can be covalently linked within the $tRNA^{Phe}$. Up to 4.5 moles of formaldehyde can be bound in the absence of Mg^{2+}. Only two of these are irreversibly bound. On the other hand, 2.5 moles of formaldehyde are bound to $tRNA^{Phe}$ in the presence of Mg^{2+}. Only one of these cannot be removed by dilution. Of the three potential linkages (G-CH_2-G, A-Ch_2-G, A-CH_2-A), the first is labile at neutral pH but stable in alkali. The others appear to be stable to the usual condition of isolation. Efforts to recover the cross-linked region from $tRNA^{Phe}$ have failed due to instability of the derivative. Although this suggests that a G-CH_2-G linkage is present, the chemical knowledge in this area is at present too limited to reach a firm conclusion. The modified tRNA retains essentially all of its acceptance activity and does not show altered gel electrophoretic properties.

To provide a reference molecular size for potential *inter*molecular linkages, we treated $tRNA^{Phe}$ with I_3^- under conditions reported to cause formation of disulfide bridges. Analysis of the product showed that the spectral peak ascribed to 4-thiouridine is indeed destroyed by I_3^-. The other sulfur-containing minor component (2-methylthio-6-isopentenyladenosine) gives rise to a new chromatographic peak. The molecular product of the 4-thiouridine, however, does not appear to be a disulfide. No thiouridine disulfide was isolatable after combined hydrolysis of RNase T_1, snake venom diesterase, and alkaline phosphatase. In addition, the characteristic spectrum of the 4-thiouridine sulfenic acid, usually obtained by alkaline cleavage of the disulfide, was not observed in alkali. From these findings, we conclude that the 4-thiouridine disulfide is not formed by I_3^-. Up to 1.8 gram-atoms of iodine were incorporated per mole of $tRNA^{Phe}$. Because of the covalent character of the iodine linkage, this product has potential value for x-ray crystallographic studies.

Recent reports from the Institut de Biologie Physico-Chimique (Paris) contain evidence for a radiation-induced cross-link between 4-thiouridine and a nonadjacent cytidine in *E. coli* $tRNA^{Val}$. We have tested our $tRNA^{Phe}$ to determine whether it can undergo this reaction. Irradiation of the $tRNA^{Phe}$ resulted in two phenomena not reported for the $tRNA^{Val}$. The early stages of reaction involved changes in the ultraviolet absorbance at 335 and 360 nm that were dependent upon the presence or absence of Mg^{2+}. In the final stages of the reaction, the spectrum was similar to that of the modified $tRNA^{Val}$ only if Mg^{2+} ion was present. In the absence of Mg^{2+}, the 360-nm absorbance rapidly decreased, while the 335-nm absorbance was the same in both the presence and absence of Mg^{2+}. This suggests that several stages of reaction must occur prior to the final linkage between thiouridine and cytidine, if such occurs.

1.2 SEQUENTIAL DEGRADATION OF NUCLEIC ACID

Mayo Uziel J. X. Khym
R. K. Fujimura Chongkun Koh
A. Jeannine Bandy

The successful application (by Khym and Uziel) of sequential degradation of tRNA prompted attempts to perform the same type of degradation on an RNA of high molecular weight (R17 RNA, mol. wt = 1.1×10^6). After modifications of the earlier procedure (viz., improved precipitation conditions and increased analytical sensitivity), we carried out 15 cycles of degradation. The results of the first experiments indicate a terminal adenosine followed by 14 cytidine residues in the major component (*ca.* 70% of the chains) and a

terminal C followed by 14 A's in the minor components. A repetition of the experiment has given qualitatively the same results. The above nucleotide sequence is at variance with published sequences of this region of the R17 RNA molecule. In addition the base analyses indicate at least two separate chains in this homogeneous phage preparation.

1.3 AUTOMATED SEQUENTIAL DEGRADATION OF RIBONUCLEIC ACIDS

Mayo Uziel J. W. Starken[1]
W. F. Johnson[1] R. A. Zingaro[2]

An instrument has been designed to perform repetitively all the steps leading to the production of free base from the vicinal hydroxyl terminus of an RNA (by the method of Khym and Uziel[3]). To minimize asynchrony, two reaction vessels are used, one for the oxidation-elimination step and one for the enzymatic removal of terminal phosphate. The instrument employs N_2 gas pressure and valving constructed of chemically inert plastics to transfer the fluid from one vessel to the other. The mechanical components have been found to perform as designed.

Two problems have arisen in the operation of the reaction vessel. The vessel-rinsing mechanism does not have the desired efficiency, so the vessel design is being investigated. The second problem involves the stability, storage, dispensing, and removal of the enzyme solution. We have solved most of these subproblems by preparation of an active insoluble alkaline phosphatase. The remaining problem is the preparation of the enzyme in a readily filterable form.

References

[1] Instrumentation and Controls Division.

[2] Present address: Department of Biochemistry, Texas A & M University, College Station.

[3] J. X. Khym and Mayo Uziel, *Biochemistry* 7, 422 (1968).

1.4 MODIFICATION OF tRNA WITH *p*-MERCURIBENZOATE

B. C. Pal K. R. Isham
L. R. Shugart M. P. Stulberg

Shugart and Stulberg[1] demonstrated that the specific recognition of $tRNA^{Phe}$ by phenylalanyl-tRNA synthetase depends upon intact dihydrouridine and/or thiouracil in the 5′ loop of the tRNA. We wish to define further this dependence by specifically treating the thiouridine-containing $tRNA^{Phe}$ with sulfhydryl reagents and examining the product for aminoacylation activity.

Experiments have been performed with model compounds. 1-Methyl-4-thiouracilyl-*p*-mercuribenzoic acid was obtained in 60% yield by treating 1-methyl-4-thiouracil (the methyl analog of 4-thiouridine) with *p*-hydroxymercuribenzoate and has been characterized by melting point, uv absorption spectra, and elemental analysis. X-ray crystallography of the complex indicates the covalent nature of the -Hg-S- bond. 1-Methyl-4-thiouracil can be regenerated from the complex by cysteine or β-mercaptoethanol. The formation of a similar mercury complex with 4-thiouridine can be followed spectrophotometrically by the loss of ultraviolet absorbance at 335 nm and a gain in absorbance at 310 nm.

2-Thiouridine was also found to react with the mercuribenzoate. In this case the peak at 273 nm in the uv absorption spectrum of 2-thiouridine at pH 7 disappears, and the spectrum in the region 270–310 nm shows a hypsochromic shift paralleling the case of 4-thiouridine.

About two-thirds of the absorbance at 337 nm in the spectrum of *E. coli* mixed tRNA comes from 4-thiouridine. On treatment of the tRNA with the mercuribenzoate, the peak at 337 nm disappears, and the spectrum shows a hypsochromic shift resulting in increased absorbance at 310 nm. This is consistent with the behavior of the model compound and is taken as evidence for the reaction of 4-thiouridine moieties in the tRNA. tRNA so treated can be dialyzed against water to remove excess reagent without altering the spectrum in the region 310–380 nm, but on dialysis against Mg^{2+} the original spectrum of the tRNA is restored, indicating the release of the tRNA-bound mercurial. In contrast, the uv absorption spectrum of the 1-methyl-4-thiouracil complex is unaltered by the addition of excess magnesium chloride. Probably 4-thiouridine is one of the binding sites for Mg^{2+} in tRNA.

Future experiments will involve the reaction of the mercurial with purified $tRNA^{Phe}$. We will determine the aminoacylation activity of the product as well as proof of the reaction by hydrolysis of the tRNA and identification of the modified base.

Reference

[1] L. Shugart and M. P. Stulberg, *Biol. Div. Ann. Progr. Rept. Dec. 31, 1968,* ORNL-4412, p. 182.

1.5 SYNTHESIS OF 2-THIOURIDINE

B. C. Pal

Although no tRNA containing 2-thiouridine or its derivatives has been sequenced so far, this substance is known to occur in mixed tRNA.[1,2] Carbon[3] obtained indirect evidence for the formation of a disulfide bond between 2-thiouridine moieties in tRNA as a result of iodine oxidation. Chambers,[4] on the other hand, was unable to prepare 2-thiouridine disulfide by iodine oxidation of 2-thiouridine. To reexamine this problem and to study the properties of 2-thiouridine, I decided to synthesize the compound by the mercury derivative method. Contrary to the previous report,[5] 2-thiouracil was found to form in quantitative yield a di(2-thiouracilyl)mercury derivative that, on treatment with 2,3,5-tri-*O*-benzoyl-1-D-ribofuranosyl chloride, yielded a small amount of the protected nucleoside, which was isolated by column chromatography on silica gel. Also, contrary to the earlier finding,[5] the yield did not improve when the reaction was carried out in the presence of mercuric bromide. The protected nucleoside undergoes debenzoylation in alkali to form 2-thiouridine in high yield.

References

[1] J. Carbon, H. David, and M. H. Studier, *Science* **161**, 1147 (1968).

[2] L. Baczynskyj, K. Biemann, and R. H. Hall, *Science* **159**, 1481 (1968).

[3] J. Carbon and H. David, *Biochemistry* 7, 3851 (1968).

[4] R. W. Chambers and V. Kurkov, *J. Am. Chem. Soc.* **85**, 2169 (1963).

[5] H. Lee and P. W. Wigler, *Biochemistry* 7, 1427 (1968).

1.6 DESALTING AND ANALYTICAL SEPARATIONS OF PURINES AND PYRIMIDINES ON SEPHADEX G-10 COLUMNS

J. X. Khym Mayo Uziel

When the carboxyl groups of a highly cross-linked dextran gel are converted to their acid form, the gel can act as a cation exchanger toward purine and pyrimidines containing basic groups, and as a result the distribution coefficients of such compounds are markedly increased over those values obtained on nonacidified dextran. At alkaline pH's these same carboxyl groups interact in an opposite manner with the acidic groups of purines and pyrimidines to lower their distribution coefficients. Choosing a proper balance between the charge on the matrix of the gel, the charges of the compounds themselves, and the adsorptive effects of the compounds for the ether groupings of the dextran gel makes possible the analytical separation of purines and pyrimidines at acidic or alkaline pH's.

It has been found recently that the "acidic" form of G-10 also can be used to desalt the nanomole amounts of purines and pyrimidines that are obtained in small-scale degradations of RNA. Initially the use of G-10 columns for this purpose was unsatisfactory because of the overlap in the elution positions of the pyrimidines with some of the components used in the degradation of RNA, such as lysine and the cetyltrimethylammonium cation (CTA). This difficulty was overcome by operating the G-10 columns in the presence of dilute acetic acid and by minimizing the amount of CTA to be removed.

During these desalting processes it was observed that less than 0.01 micromole of purine or pyrimidine could be qualitatively detected in the effluents from the G-10 columns; this was done by monitoring the column fractions with an ordinary Isco ultraviolet analyzer. This suggests that quantitative analysis of nanomole amounts of purine and pyrimidine, even in the presence of large amounts of salt, is possible if a finer mesh Sephadex bead were used in an analytical-type column; an instrument more sensitive than Isco would be needed to quantify the column effluents. Such a system could eliminate a time-consuming step in the present procedure wherein desalted samples are concentrated prior to analysis by high-resolution chromatography.

1.7 PROPERTIES OF T2 mRNA

Elliot Volkin M. Helen Jones

We have reported[1] that the messenger RNA synthesized early or late after T2 infection has an average nucleotide length of about 600 residues and sediments with a mode of about 12S. In addition, all four bases were observed at both the 5′ and 3′ termini, with only 5′ monophosphate rather than 5′ triphosphate groups at the presumed initiation ends. These results indicate that a large percentage of the RNA molecules had undergone some degree of hydrolysis, especially at the initiation end. The rapid turnover of phage mRNA is well established. In 1959[2] we had found, and many others have since discovered, that the antimetabolite chloramphenicol prevents turnover (or degradation) of phage mRNA but apparently does not prevent synthesis of this RNA. We have now analyzed the T2-specific mRNA made in the presence of chloramphenicol. The

presence of the 5′ triphosphate termini (probably containing the purines adenine and guanine) can be clearly demonstrated in these molecules. However, surprisingly, the average sedimentation rate of this mRNA is only about 8S, and the average nucleotide length of the polymers (determined by end-group analysis) is only about 250 residues. It seems that the synthesis of the phage mRNA molecules in the presence of chloramphenicol is initiated properly but is terminated prematurely.

References

[1] E. Volkin and W. S. Riggsby, *Genetics* **61**, 409–28 (1969).

[2] L. Astrachan and E. Volkin, *Biochim. Biophys. Acta* 32, 449–56 (1959).

1.8 UPTAKE OF NUCLEIC ACIDS BY HUMAN CELL LINES

Elliot Volkin James D. Regan
M. Helen Jones

Studies have been initiated which we hope to be the forerunners of experiments for transferring genetic information from one human cell line to a different human cell line. We have found that DNA from PGLC (lymphoblastoid) cells is incorporated in reasonably large quantity into HS (skin) cells in tissue culture. The incorporation takes place in a roughly linear way for 4 to 6 hr. Incorporated DNA is totally retained for at least 6 hr more (4-hr pulse, 6-hr chase experiment) and is incorporated as some kind of polynucleotide. Double-labeling experiments to demonstrate the latter conclusion also showed that PGLC RNA was nicely incorporated by the HS cells. The incorporation rate and stability of the RNA in the recipient cells were similar to those of incorporated DNA. One facet of the RNA studies that seems to be especially interesting is the fact that the RNA incorporated is not representative of the total RNA to which the cells were subjected. That is, the nucleotide composition of total PGLC RNA has an AU/CG ratio of 0.80, but the AU/CG ratio of the incorporated RNA is about 1.3. It is interesting that the corresponding AT/CG ratio of human cell DNA is about 1.4.

1.9 PURIFICATION OF NATIVE PHAGE λ DNA

C. G. Mead

In order to develop an assay for an enzyme postulated to be involved in the recognition of complementary nucleotide sequences in DNA molecules, it was necessary to obtain DNA preparations that were completely native. The criterion of nativeness in this case was the ability of DNA to pass through a nitrocellulose filter when dissolved in 6XSSC. It was noted that different preparations of phage λ DNA varied greatly in their ability to pass through nitrocellulose filters. For some purposes the DNA had to be cross-linked with nitrogen mustard, which further reduced the ability of the DNA to pass through the filters.

The fraction of DNA that adsorbed to nitrocellulose filters could not be removed by cesium chloride density gradient centrifugation or by chromatography on hydroxyapatite. It was found that this fraction could, however, be removed by partition chromatography on the reverse-phase column described by Kidson.[1] This fraction that adsorbed to the filters was eluted behind the native DNA and ahead of denatured DNA. This DNA is therefore most probably partially single stranded. The amounts of this DNA varied from 10 to 50% of the preparations.

Reference

[1] C. Kidson, *Biochemistry* 8, 4376–82 (1969).

1.10 BIOCHEMICAL ANALYSIS OF RECOMBINANT MOLECULES OF BACTERIOPHAGE T5 DNA

R. K. Fujimura Helen T. Tucker

We have been analyzing a repair that involves the synthesis of a short stretch of oligonucleotide to link a broken section. Our assumption is that a similar mechanism is required for recombination of DNA molecules. We have obtained evidence that such breakage and repair occurs continuously among T5 DNA molecules in vivo. We have been trying to perfect techniques whereby such repaired sections can be subjected to a biochemical test for linkage, that is, analysis of transfer of "nearest-neighbor phosphorus" from the end of a repaired section to the end of a preexisting strand. For this purpose the T5 DNA extracted must be nonreplicated DNA and free of *E. coli.* DNA. This was accomplished by CsCl density gradient centrifugation and hybridization to T5 DNA–nitrocellulose powder. The purity of DNA was tested by hybridization on T5 DNA and *E. coli* DNA nitrocellulose membranes in formamide. The purified DNA was degraded to mononucleotides, and the trans-

fer of phosphorus to bromodeoxyuridylic acid was analyzed by a combination of chromatography in Bio-gel DM-2 and thin-layer electrophoresis. Such analyses have shown that the repaired section formed in the absence of T5 DNA polymerase has a nucleotide composition the same as that of whole T5 DNA and a chain varying in average length from about 30 to 200. It is not clear at the moment whether this difference is due to inaccuracies in analytical technique or variability in biological conditions.

Recently we have obtained two *E. coli* DNA polymeraseless mutants – one isolated at John Cairns' laboratory and obtained through John Boyle, the other obtained from S. Kondo's laboratory. We are analyzing T5 phage-infected cells of these mutants to find the role of T5 DNA polymerase and *E. coli* DNA polymerase in repair and recombination by the methods described above.

1.11 ON STRUCTURE OF BACTERIOPHAGE T5 DNA

R. K. Fujimura Helen T. Tucker

When bacteriophage T5st is centrifuged in an alkaline sucrose gradient, its DNA will separate into four or five peaks. Abelson and Thomas have come to the conclusion that the strand that has the fragment I has an additional piece, IV (numbered in order of decreasing size). A recent report by Bujard, however, shows that for $T5^+$ DNA one of the strands is intact. If the largest piece is an intact strand, it should be 50% of the whole DNA. For T5st DNA, fragment I from our alkaline sucrose gradient centrifugation was always less than 50% of the whole. However, when the fractionated DNA fragments were hybridized against one another on nitrocellulose membranes, all the smaller fragments hybridized best to I, indicating I is complementary to the rest of the fragments. This apparent inconsistency may be due to masking of a piece complementary to some strand other than I by neighboring pieces or to breakdown of some of the I. Thus the smaller fragments were hybridized to I and II in the presence of excess of I. It was found, indeed, that there are fragments that hybridize better to II than to I. It is not yet clear whether these fragments are broken pieces of I or some other fragments.

J. P. Breillatt, Jr., has been kindly providing us with milligram quantities of T5 DNA fragments which he had fractionated by means of zonal centrifugations using a rotor specifically designed for a fragile substance such as DNA.

1.12 THE ASSOCIATION OF SHORT OLIGORIBONUCLEOTIDES WITH DENATURED DNA AT LOW TEMPERATURES IN THE PRESENCE OF Mg^{2+}

S. K. Niyogi

In the absence of Mg^{2+}, a chain length of at least ten ribonucleotides is needed to form a ribonuclease-resistant complex with denatured DNA. In the presence of 0.01 *M* Mg^{2+}, 8-mers and longer can form a detectable complex with denatured DNA. Oligonucleotides, 2-mers to 7-mers, from T2 and T7 RNA's were tested for their annealing to denatured DNA on membrane filters at various low temperatures ranging from 0 to 15°C. The solvent used was 0.5 *M* KCl, 0.01 *M* $MgCl_2$, 0.01 *M* Tris, pH 7.8, and ribonuclease treatment, washings, etc., were performed in this solvent at 0°C. Small amounts of annealing could be detected starting at 3-mers, with the efficiency of annealing increasing with the chain length of the oligonucleotide.

Oligonucleotides, 2-mers to 12-mers (with free 3′-OH end groups), from both T2 and T7 RNA's were tested for stimulation of RNA synthesis with all four ribonucleoside triphosphates on both native and denatured DNA templates. At 0°C, oligomers as short as 3-mers stimulate RNA synthesis when denatured DNA is used as template. The amount of stimulation increases with the chain length of the oligonucleotide. At higher temperatures only the longer oligomers stimulate the reaction.

1.13 *N*-FORMYLMETHIONYL TRANSFER RNA IN MITOCHONDRIA FROM *NEUROSPORA*

J. L. Epler

Although the mechansim for the initiation of protein synthesis in bacteria appears to involve *N*-formylmethionyl transfer RNA (fMet-tRNA), little is known of the initiation process in higher organisms. Since the mitochondria of *Neurospora* contain a protein-synthesizing system with components distinct from those of the cytoplasm, we have examined both mitochondrial and cytoplasmic extracts for fMet-tRNA. A Met-tRNA from the mitochondria can be formylated with an *E. coli* extract, but the corresponding cytoplasmic tRNA cannot. This fMet-tRNA is chromatographically distinct from another mitochondrial Met-tRNA that cannot be formylated. These are distinct from the cytoplasmic Met-tRNA and *E. coli* tRNA's.

The mitochondrial methionyl-RNA synthetase is highly specific, aminoacylating only the mitochondrial

tRNA's. Similarly, the mitochondrial extracts contain a transformylase that reacts with only the mitochondrial fMet-tRNA. Thus, it appears that mitochondrial protein synthesis involves a formylated methionine, presumably as an initiator in analogy to the bacterial system. The mechanism of initiation in the cytoplasmic protein-synthesizing system is being investigated.

1.14 INDUCIBLE tRNA'S AND AMINOACYL-RNA SYNTHETASES

W. E. Barnett S. A. Fairfield[1]
Bonnie J. Reger

At the present time there is much speculation that tRNA's play a major role in cell regulatory phenomena during differentiation, oncogenesis, etc. It is not difficult to envision a mechanism in which the availability of a single species of tRNA determines the translation of a group of polycistronic messages. There is at present, however, no direct evidence that such a mechanism exists in nature. As an approach to this issue, we have established a system in which one can experimentally manipulate the cellular complement of tRNA's by the expedient of altering the cell's energy metabolism. It is anticipated that the system will permit an experimental evaluation of the notion that new species of tRNA's must be made available prior to the synthesis of major new classes of proteins (enzymes).

Specifically we have shown that exposure of dark-grown *Euglena* cells to light results in the appearance of at least six new chromatically unique species of tRNA. Studies with drugs and mutant strains indicate that light induction of the new tRNA's is dependent upon the cell's ability to grow photosynthetically, thus implicating them in the synthesis of some component(s) of the photosynthetic apparatus. We have also found that light induces the appearance of at least one new synthetase. This is puzzling, since it is not apparent why additional synthetases are required.

A great deal of effort has been put into chloroplast isolation and attempts to establish that the light-inducible species of tRNA are localized in the organelle. All results so far indicate that they are not.

Reference

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

1.15 INDUCTION OF AMINOACYL RNA SYNTHETASES IN *EUGLENA*

Bonnie J. Reger J. L. Epler
W. E. Barnett Linda K. Mann

The aminoacyl synthetases and tRNA's of *Neurospora crassa* mitochondria have been characterized by Barnett *et al.* They found the mitochondria to contain a full complement of both aminoacyl synthetases and tRNA's and presented evidence that three of these macromolecules were unique to this organelle and distinct from those found in the cytoplasm of *Neurospora.*

Chloroplasts are known to contain DNA, RNA, and ribosomes. Since chloroplasts also synthesize protein in vitro, they obviously contain aminoacyl RNA synthetases and tRNA's. The synthetases and tRNA's of this organelle have not yet been directly characterized. Barnett *et al.* have found an induction of chromatographically new species of tRNA in *Euglena gracilis* strain B when these cells were grown in the light rather than in the dark. Parallel studies with a bleached mutant of *Euglena* (W_3BUL) have shown that the induction of the new tRNA's was dependent on chloroplast formation rather than any effect of light per se. The present study is concerned with characterizing induced species of aminoacyl RNA synthetases in *Euglena* – the wild type and the mutant. Attempts are being made to isolate the chloroplasts in large quantities so that the synthetases and tRNA's can be characterized directly and thus to define the translational apparatus (*i*) of organelles.

Aminoacyl RNA synthetases from light-grown *Euglena gracilis* strain B and mutant *Euglena* W_3BUL have been partially purified by ammonium sulfate fractionation followed by hydroxyapatite and DEAE-Sephadex (A-25) column chromatography. Preliminary results indicate that light-grown wild-type *Euglena* contains more than one aminoacyl RNA synthetase species for at least isoleucine and phenylalanine. In contrast, the bleached mutant contains a single species for most amino acids tested. Other results suggest that light induces the formation of new species of synthetases in wild-type *Euglena.* Further characterization of the aminoacyl RNA synthetases of light- and dark-grown *Euglena* and the mutant *Euglena* is currently under way.

1.16 IN VIVO DIRECTION OF PROTEIN SYNTHESIS BY ADDED MESSENGER RNA IN *PHYSARUM POLYCEPHALUM*

A. A. Francis Peter Pfuderer

Experimental material can be injected directly into the cytoplasm of the slime mold *Physarum polycephalum* by using the appropriate micropipet. This method bypasses the permeability problem encountered when macromolecules are introduced in other in vivo systems. The synthesis of tobacco mosaic virus (TMV) coat protein has been shown by D. G. Humm[1] to occur in *Physarum* which has been injected with isolated TMV RNA.

We have been investigating the potential of this system as an in vivo assay for heterologous messenger RNA. In our hands a volume of about 3 to 5 μl containing TMV RNA and ^{14}C-threonine can be easily injected into the cytoplasm of the mold. After the injection the mold is allowed to stand for 3 to 4 hr, after which it is homogenized in 0.25 *m* sucrose and centrifuged at $10^5 \times g$ for 30 min. The clear yellow supernatant is then incubated with antibody to the TMV protein, followed by examination for the radioactive antibody protein precipitate. We have succeeded in obtaining immunologically identifiable TMV protein in this manner. The application of this procedure for use as a quantitative assay for messenger RNA appears limited at this time, since the range from "no recoverable precipitate" to "saturation of the system" is only from about 3 to 10 μg of injected RNA. The qualitative use of this system, however, may be valuable when as much as 10 μg of isolated messenger RNA is available for assay. We are currently applying this system to qualitatively identify messenger RNA isolated from rabbit reticulocytes.

Reference

[1] Personal communication.

1.17 CHANGES IN LACTIC DEHYDROGENASE WITH AGE OF MICE

Peter Pfuderer Bill Craig[1]

We are investigating the effects of age on the protein coding and synthesizing apparatus by examining changes in a particular protein with age. Aging could very logically be primarily due to damage somewhere in the protein-synthesizing machinery resulting in an inefficient organism. It has already been shown that an increase in the frequency of errors during protein synthesis accelerates aging,[2,3] and many workers have recorded changes in the LDH patterns of tissues with age.

We have utilized a gel-electrophoresis system powered by an ORTEC pulsed power supply, combined with a Polaroid camera to take pictures of the gel patterns. We have also utilized a Beckman Analytrol, fitted with film densitometer attachment normally used to scan uv runs in the ultracentrifuge, to develop an extremely sensitive analytical method of determining absolute changes in the LDH isoenzyme pattern. Shifts in the subbands of the five major isozymes from one tissue to another are readily detected and quantitated. We have used this tool to survey the shift of isoenzyme patterns of $BC3F_1$ mice with age. Preliminary results have indicated a 40% decrease in brain LDH and a 20% decrease in kidney LDH in these mice with increasing age. Little change was observed in the other tissues examined. We have also found a general decrease in the amount of the M-type monomer with age in almost all tissues examined thus far. In the mouse skeletal muscle, four new LDH-5 isozyme bands were found in the old animals. This is indicative of a new type of M monomer being formed in these old animals.

All of the above results are consistent with the hypothesis that new mutant forms of the M monomer of LDH, both active and inactive, are being produced in the tissues of older animals. We intend to further verify this hypothesis and to search for the biochemical mechanism causing the change.

References

[1] ORAU student loanee.

[2] R. Holliday, *Nature* 221, 1224 (1969).

[3] B. J. Harrison and R. Holliday, *Nature* 213, 990 (1967).

1.18 STUDIES OF RNA POLYMERASE FROM *E. COLI*

Audrey L. Stevens

Further investigations have been carried out on the 5′-terminal nucleotide sequences of the RNA chains made with highly purified RNA polymerase from *E. coli*. Using native T7 DNA as a template, RNA molecules are labeled with γ-^{32}P-ATP and ^{3}H-ribo-

nucleoside triphosphates as substrates. The RNA chains are isolated and degraded with T1 ribonuclease and spleen phosphodiesterase. The products of the degradation are γ-^{32}P-ATP containing oligonucleotides from the 5′ termini of the RNA chains starting with ATP and 3′ nucleotides. The results show that 90% of the RNA chains starting with ATP start with pppA-U-G and pppA-C-(U, C, or A)-G. The amount of each 5′-terminal sequence is dependent on the ionic strength (KCl concentration) of the reaction mixtures. At high ionic strength (0.3 *M* KCl), 66% of the chains start with pppA-U-G. That a large number of the RNA chains do start with AUG, the *N*-formylmethionine codon, shows that the *E. coli* polymerase must recognize the codon as a signal for RNA chain initiation.

1.19 MESSENGER-RNA- AND tRNA-DEPENDENT SYNTHESIS OF BIOLOGICALLY ACTIVE PROTEINS IN VITRO

Helen Sellin G. David Novelli

Our aim is to develop an in vitro mRNA- and tRNA-dependent bacterial system capable of synthesizing biologically active proteins. This system will be used with synthetic and natural nucleic acid polymers and tRNA's from a variety of sources.

Endogenous mRNA is eliminated by a Nirenberg preincubation of the crude extract. Endogenous tRNA is much more difficult to completely eliminate from the ribosomes, but two methods for producing tRNA-free ribosomes have been developed. The resulting system for poly-U-directed synthesis of polyphenylalanine is completely dependent on added mRNA and tRNA. RNA from T4-infected cells is also capable of directing incorporation of radioactive amino acids in this system. Now that we have achieved purified components a DNA-dependent system will be included to provide constant regeneration of mRNA, and we will work toward synthesis of a biologically active protein.

1.20 ISOLATION OF AMINOACYL-RNA SYNTHETASE:tRNA COMPLEXES

J. G. Farrelly J. W. Longworth
M. P. Stulberg

We have continued our studies[1] of the conformational changes taking place upon the interaction of phenylalanyl-RNA synthetase and $tRNA^{Phe}$ from *E. coli*. The techniques of fluorescence spectroscopy, optical rotatory dispersion, and circular dichroism were used in the past with limited success. We therefore turned our efforts toward isolating a stable enzyme-tRNA complex in order to examine optical properties of a more defined and stable system.

We have been able to isolate stable complexes of synthetase and tRNA by centrifugation in sucrose gradients. The complexes, which dissociated at pH 6.5 and above, were stable between pH 5.0 and 6.0 and were stoichiometric for enzyme (180,000 mol. wt) and tRNA. Fractions from the gradients were monitored for presence of complex by absorbancy at 254 nm and by the ability to form phenylalanyl-$tRNA^{Phe}$ upon the addition of ATP and phenylalanine. Quenching of tryptophan fluorescence of the enzyme upon the addition of $tRNA^{Phe}$ was also utilized to follow complex formation. The intensity of fluorescence of the enzyme varied as a function of pH with a p*K* of 5.3, and the degree of quenching, upon addition of $tRNA^{Phe}$, was constant between pH 5.0 and 6.0. Experiments using other purified tRNA species indicated that the quenching of fluorescence is specific for $tRNA^{Phe}$. We are presently designing fluorescence experiments in order to determine association constants for the enzyme-tRNA complex. Circular dichroism is also being utilized in an effort to correlate complex formation with conformational changes.

Reference

[1] J. G. Farrelly *et al., Biol. Div. Ann. Progr. Rept. Dec. 31, 1968,* ORNL-4412, p. 181.

1.21 SPLEEN EXONUCLEASE ACTIVITY ON tRNA

K. R. Isham M. P. Stulberg

We have previously reported on our efforts to use purified spleen 5′ exonuclease for sequential hydrolysis of $tRNA^{Phe}$.[1] These studies are intended to further elucidate the recognition site in $tRNA^{Phe}$ for phenylalanyl-RNA synthetase.

In addition to tRNA, other species of RNA which resist enzymic hydrolysis are ribosomal RNA and R17 viral RNA. Experiments on the action of the exonuclease on synthetic polymers indicate that poly G and poly C are highly resistant to hydrolysis, whereas poly U and poly A are easily hydrolyzed. From these

experiments and others with polynucleotides of defined base content, we have tentatively concluded that the exonuclease cannot hydrolyze RNA containing high proportions of guanine and cytosine. Therefore, tRNA which contains G- and C-rich 5′ termini and R17 viral RNA which contains a pppG-G-G-C 5′ terminus would be resistant to attack. Our final experiments involving this exonuclease will be to determine the kinetic nature of the G and C resistance and also to determine the effect of pH, if any, on the hydrolysis of poly G and poly C.

Reference

[1] K. R. Isham and M. P. Stulberg, *Biol. Div. Ann. Progr. Rept. Dec. 31, 1968*, ORNL-4412, p. 182.

1.22 AN INVESTIGATION OF CYTOKININ, tRNA, AND SENESCENCE IN WHEAT LEAVES

M. P. Stulberg K. R. Isham
L. R. Shugart L. L. Triplett
A. H. Haber

The instigation for this project was observations by others that cytokinin delays senescence in wheat leaves and that tRNA lacking cytokinin is much less efficient in promoting protein synthesis in *E. coli*. We are postulating, on the basis of these observations, that perhaps a correlation exists between cytokinin contained in tRNA and its action in the phenomenon of senescence in wheat.

We have grown and harvested eight-day wheat plants and separated the growing section from the nongrowing section of the leaf. After careful purification of extracted tRNA from the two sections, we have determined that the gross cytokinin levels in the tRNA are approximately equal in both growing and nongrowing (programmed for senescence) tissues. We shall now examine individual tRNA's from each tissue to determine content of cytokinin and incorporation of cytokinin precursors in response to cytokinin treatment of the tissue. An interesting result has been observed when the tRNA's from growing and nongrowing tissues were subjected to reversed phase 2 chromatography.[1] There is a remarkable qualitative difference in the $tRNA^{Phe}$ pattern between the two tissues. One can speculate on the basis of chromatographic behavior that the differences observed could be due to differences in cytokinin content.

Reference

[1] J. F. Weiss and A. D. Kelmers, *Biochemistry* **6**, 2507 (1967).

1.23 FUNCTION OF METHYL GROUPS IN TRANSFER RNA

L. R. Shugart K. R. Isham
Barbara H. Chastain E. C. Hise III[1]
M. P. Stulberg

Although the presence of methyl groups in tRNA has been known for a considerable time, their function has not been established. It has been demonstrated by others that methionine serves as the donor for the methyl groups in tRNA and that the tRNA molecule is methylated enzymatically after the formation of the polynucleotide. Experiments from several laboratories on the methylation of normal and undermethylated tRNA, using both homologous and heterologous methylase preparations, have been interpreted to mean that the susceptibility of tRNA to methylation involves not only a specific polynucleotide configuration within the tRNA molecule but also a high degree of enzyme selectivity as to the substrate and its source.

Shugart *et al.*[2] have shown that undermethylated $tRNA^{Phe}$ preparations differ from the normally methylated species by several criteria and further that upon in vitro methylation of undermethylated tRNA with methylases free of RNase, an increase in several aminoacylation activities occurred.[3] These data suggest that the degree of methylation of the tRNA affects the aminoacylation activity of the molecule. To fully understand how the methyl group is involved, a study of the interaction of the tRNA and the enzymes has been undertaken in a completely defined environment utilizing highly purified undermethylated tRNA species and methylating enzymes. Such a study is being approached in a two-step manner. At present we are isolating and purifying various methylases from bacterial and plant material. Undermethylated tRNA species from the RC^{rel} strain of *E. coli* are being purified by combining BD-cellulose column chromatography with reversed-phase column chromatography. Once these purified materials are available in sufficient quantities, a detailed characterization of the methylases will be made, and the biological and physical properties of the undermethylated tRNA will be determined upon remethylation in vitro.

References

[1] ORAU student loanee.

[2] L. R. Shugart, G. D. Novelli, and M. P. Stulberg, *Biochim. Biophys. Acta* **157**, 83 (1968).

[3] L. R. Shugart, Barbara H. Chastain, G. D. Novelli, and M. P. Stulberg, *Biochem. Biophys. Res. Commun.* **31**, 404 (1968).

1.24 A STUDY OF tRNA IN WHEAT AND ITS ROLE IN CELLULAR REGULATION

L. R. Shugart Barbara H. Chastain
A. H. Haber

It has been suggested that transfer RNA may be involved in cellular regulation,[1] and one line of evidence for this theory would be the observation of a change in the distribution of certain tRNA species during differentiation.[2] Originally this work was undertaken to obtain heterologous enzymes and tRNA's to be used in conjunction with our study on the function of methyl groups in tRNA of *Escherichia coli.* Our preliminary results, obtained from a comparison of the activities of enzyme preparations from wheat and *E. coli* on homologous and heterologous tRNA's, indicated that there are significant quantitative differences between the tRNA's of wheat embryo tissue and eight-day-old wheat shoots. Furthermore, qualitative differences have also been found for two tRNA's (methionine and phenylalanine) as shown by cochromatography of precharged tRNA's on reversed-phase columns.

References

[1] G. D. Novelli, *J. Cell. Physiol.* Suppl. 1 **74**, 121–48 (1969).

[2] Vold and Sypherd, *Proc. Natl. Acad. Sci. U.S.* **59**, 453–58 (1968).

1.25 FUNCTION OF 4-THIOURIDINE IN TRANSFER RNA

L. R. Shugart Barbara H. Chastain

The 5′ loop area of (*E. coli*) $tRNA^{Phe}$ contains two dihydrouridine moieties and one 4-thiouridine moiety. When these are selectively reduced with $NaBH_4$, there is a concomitant loss of $tRNA^{Phe}$ to be aminoacylated.[1] Other (*E. coli*) tRNA molecules known to contain these nucleotides are not similarly affected. These results, along with those of Stulberg and Isham,[2] suggest that the non-hydrogen-bonded 5′ loop area of (*E. coli*) $tRNA^{Phe}$ contains the information for synthetase recognition.

Our current experiments have been directed toward the *selective* modification of only 4-thiouridine in tRNA and toward determining whether a loss of aminoacylation activities occurs after modification. Those types of modifications performed to date have been

1. phototransformation of nonstructured tRNA in an organic solvent by irradiation at 3300 Å, and
2. chemical transformation using H_2O_2 and *N*-ethylmalimide.

Preliminary data indicate that a loss of 4-thiouridine from tRNA does not affect the ability of the molecule to be aminoacylated.

References

[1] L. R. Shugart and M. P. Stulberg, *J. Biol. Chem.* **244**, 2806 (1969).

[2] M. P. Stulberg and K. R. Isham, *Proc. Natl. Acad. Sci. U.S.* **57**, 1310 (1967).

1.26 PHENYLALANYL-tRNA SYNTHETASE OF *NEUROSPORA CRASSA* CYTOPLASM: CONDITIONS THAT AFFECT THE CHARGING AND DISCHARGING OF HETEROLOGOUS tRNA

P. O. Ritter[1] F. J. Kull[2] K. Bruce Jacobson

Phenylalanyl-tRNA synthetase (Syn^{Phe}) from *N. crassa* catalyzes the formation of Phe-$tRNA^{Val}$ (*E. coli*). This is a highly unusual reaction because an error in recognition is made. The reaction between an aminoacyl-tRNA synthetase and the appropriate amino-acid-specific tRNA is normally highly specific even when the synthetase and the tRNA are from different sources. By studying the interactions between Syn^{Phe} (*N. crassa*) and $tRNA^{Val}$ (*E. coli*) we hope to gain some insight into the mechanism of enzymatic aminoacylation and to learn something about enzyme recognition sites on the tRNA molecule.

The aminoacylation of $tRNA^{Val}$ (*E. coli*) by Syn^{Phe} (*N. crassa*) is extremely sensitive to assay conditions. In Tris buffer under optimum conditions the final yield of Phe-$tRNA^{Val}$ is a function of enzyme concentration, and aminoacylation is incomplete even at very high enzyme concentrations. At low enzyme concentrations the final yield of Phe-$tRNA^{Val}$ is increased tenfold by 20% dimethyl sulfoxide. Ethanol (10%) also stimulates (2.3-fold) aminoacylation, whereas NaCl, NH_4Cl, and 2-mercaptoethanol are all inhibitory. When cacodylate buffer is used in place of Tris buffer, the pH optimum for aminoacylation is shifted from above 8.1 to 6.3, and various kinetic properties of the aminoacylation reaction are changed. At low enzyme concentrations over ten times more Phe-$tRNA^{Val}$ is formed in cacodylate buffer than in Tris buffer. Dimethyl sulfoxide (20%) and all concentrations of 2-mercaptoethanol, ethanol, NaCl, and NH_4Cl inhibit aminoacylation in cacodylate buffer. These results emphasize the fact that buffers are not inert compounds which act only to maintain a relatively constant pH in a reaction mixture. The mechanism of enzymatic aminoacylation may actually be different in different buffers.

Dimethyl sulfoxide, ethanol, 2-mercaptoethanol, and NaCl have no effect on the rate of deacylation of Phe-$tRNA^{Val}$ (*E. coli*) under conditions that are optimum for the aminoacylation of $tRNA^{Val}$ (*E. coli*) by Syn^{Phe} (*N. crassa*). Therefore, the effects that these compounds have on the aminoacylation of $tRNA^{Val}$ must be due to direct effects on the aminoacylation process itself.

Since Syn^{Phe} (*N. crassa*) can form Phe-$tRNA^{Val}$, we examined the ability of this enzyme to carry out the reverse reaction. Syn^{Phe} deacylates not only Phe-$tRNA^{Val}$ but also Val-$tRNA^{Val}$. The deacylation of the former requires AMP and pyrophosphate, the known substrates, but deacylation of the latter requires neither. Thus we propose that Syn^{Phe} recognizes Val-$tRNA^{Val}$ exclusively by the specific properties of the tRNA and that the valine ester bond becomes labile. Then the amino acid site of the enzyme rejects valine, causing it to be released from the tRNA.

We conclude that the reaction of an aminoacyl-tRNA synthetase with heterologous tRNA is extremely sensitive to the environment in ways that it is not when the enzyme reacts with homologous tRNA.

References

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1.27 PROPERTIES OF CYTOPLASMIC PHENYLALANYL-tRNA SYNTHETASES OF *NEUROSPORA CRASSA*

F. J. Kull[1] K. Bruce Jacobson

Phenylalanyl-tRNA synthetase (Syn^{Phe}) has been studied since it makes a mistake in recognizing tRNA; namely, it will charge phenylalanine onto (*E. coli*) $tRNA^{Val}$. In an attempt to understand the interaction of this class of enzymes and tRNA, we have studied this enzyme by first purifying it and then characterizing its kinetic and physical properties.

The enzyme was purified by conventional technics; propylene glycol and mercaptoethanol were required to prevent denaturation. When chromatographed on hydroxylapatite three zones of enzyme activity were resolved, A, B, and C. Considerable attention was then devoted to the properties of these three forms so that we could better understand the basis for their differences.

The three enzyme forms are all the same size, as defined by their sedimentation rates in sucrose density gradients. The C form was obtained as a homogeneous protein with molecular weight of approximately 180,000.

The A and C forms of Syn^{Phe} were dissimilar in the following ways: (1) In the absence of mercaptoethanol the half-life of the C form was one-tenth that of the A form. (2) Antibodies to the C form were able to inactivate the A form, but twice as much was required as for an equivalent amount of the C form. (3) The activity of both was increased by 15% propylene glycol, but the A form was stimulated only one-half as much as the C form. (4) The activity of the A form did not respond to the presence of magnesium ion as much as the C form did. (5) The pH optimum for both was 9.5, but at suboptimal pH's the amount of activity varied differently.

The A and C forms of Syn^{Phe} had very similar K_m's for ATP and phenylalanine. Their K_m's for tRNA differed by a factor of 2 to 3, and all three forms, A, B, and C, make the same "mistake" in that they charge *E. coli* $tRNA^{Val}$ with phenylalanine. Under limiting conditions caused by Tris buffer, the A form can charge twice as much *E. coli* $tRNA^{Val}$ as the C form.

A striking inhibitory effect by inorganic pyrophosphate was observed using *E. coli* $tRNA^{Val}$ as substrate, and a marked stimulation of the enzyme resulted from the presence of the enzyme inorganic pyrophosphatase. This inhibition by low concentrations of pyrophosphate may be a general property of reactions involving enzyme and tRNA from heterologous sources.

We conclude that the multiple forms of Syn^{Phe} need to be taken seriously. Further experiments will be required to determine the basis of their difference.

Reference

[1]Present address: Department of Biology, State University of New York, Binghamton.

1.28 MERCAPTOETHANOL: REACTION WITH CACODYLIC ACID

K. Bruce Jacobson J. B. Murphy

In the course of studying the catalytic properties of an enzyme in a solution containing mercaptoethanol and cacodylate buffer, the enzyme's behavior appeared to be anomalous. The possibility of a reaction of cacodylate ion with mercaptoethanol was tested and confirmed.

A mixture of 0.05 *M* 2-mercaptoethanol in an aqueous solution of 0.1 *M* potassium cacodylate pH 5.5 results in the following phenomena:

1. The sulfhydryl concentration rapidly decreases to one-half the original value and remains at this level at ten times higher concentration of cacodylate.
2. The acid function of the cacodylate molecule is decreased and is entirely lost in an excess of mercaptoethanol.
3. The reaction kinetics are those of a second-order reaction.
4. An increase in absorption of ultraviolet light (230–260 nm) occurs, and the increase parallels the loss of sulfhydryl groups.
5. The uv absorption of the product is lost irreversibly at pH 8.

These observations indicate that cacodylate and mercaptoethanol react to form a product that has no free sulfhydryl group, has no free acid, has a structure that absorbs ultraviolet light, and has a short half-life at a slightly alkaline pH.

Other sulfhydryl compounds commonly used in enzymology and found in nature (cysteine, glutathione) also react with cacodylate in much the same way that mercaptoethanol does.

The significance of these sulfhydryl-cacodylate products should be evaluated by testing their effect on enzyme reactions. There may be further significance since cacodylic acid is used in large amounts as a defoliant.

1.29 STUDIES ON A MODIFICATION OF $tRNA^{Tyr}$ ASSOCIATED WITH A SUPPRESSOR GENE IN *DROSOPHILA MELANOGASTER*

D. R. Twardzik E. H. Grell K. Bruce Jacobson

It has been shown in microorganisms that a genetically altered tRNA species can function as a suppressor of both nonsense and missense mutations. Strains that can suppress both ochre and amber mutations have been shown to contain tRNA's capable of introducing an amino acid into protein in response to a nonsense codon. In *E. coli,* a one-base change in the anticodon region of tyrosyl tRNA allows it to recognize the amber codon, UAG, with subsequent polypeptide chain elongation.

A comparison of tRNA's from a wild inbred strain (Samarkand) of *D. melanogaster* with a suppressor mutant, $su(s)^2$, for vermilion eye color was made on a reverse phase 2 chromatographic system. Optimum pH, ATP, and Mg concentrations were determined for the aminoacylation of phenylalanine, leucine, serine, and tyrosine tRNA. Similar isoaccepting patterns were seen for phenylalanyl, leucyl, and seryl tRNA, but, on the other hand, cochromatography of tyrosyl tRNA demonstrated a significant change in its isoaccepting pattern. Wild-type $tRNA^{Tyr}$ was separated into two major isoaccepting forms designated peak 1 and 2, in order of elution. In the mutant homozygous for $su(s)^2$, peak 2 was absent or greatly reduced and peak 1 was proportionally larger. Reciprocal crosses were made between wild-type males +/Y and females homozygous for the $su(s)^2$ gene and between wild-type females +/+ and males hemizygous for $su(s)^2$. The progeny were then identified and separated by genotype. Transfer RNA^{Tyr} from both heterozygous females, $su(s)^2/+$, and +/Y males chromatographed as wild type, indicating that the gene responsible for the change in $tRNA^{Tyr}$ is recessive and sex linked. Some of peak 2 of $tRNA^{Tyr}$ is present in hemizygous males $su(s)^2/Y$, indicating a dose effect.

Since genetic studies have shown that the su(s)^2 locus is also sex linked and recessive, a correlation between the su(s)^2 locus and the gene causing the change in $tRNA^{Tyr}$ exists. An additional demonstration of a direct correlation was made by inducing another suppressor for vermilion eye color at the same locus with ethyl methanesulfonate. Peak 2 of $tRNA^{Tyr}$ is also absent in the EMS-induced suppressor, su(s)^{e1}, as is the case in the su(s)^2 mutant.

No conformational differences between the peaks of $tRNA^{Tyr}$ were apparent as tested by heating and quick cooling the tRNA; G-100 chromatography showed them to be the same size. Charging wild tRNA with su(s)^2 enzyme and su(s)^2 tRNA with wild enzyme did not affect the initial observations. A T1 RNase digest of $tRNA^{Tyr}$ from wild and mutant *Drosophila* suggested similar terminal 3′-OH oligonucleotides.

Primer-dependent polynucleotide phosphorylase from *Micrococcus lysodeikticus* is being used to synthesize the codons for tyrosyl-tRNA, UAU and UAC. Codon recognition studies of $tRNA^{Tyr}$ from the wild-type and the suppressor mutant in response to the tyrosine codons and to the amber (UAG) and ochre (UAA) triplets are planned.

1.30 ANALYSIS OF POSSIBLE TRANSLATIONAL FACTORS IN AGING

J. M. Frazer Wen-Kuang Yang

Of the many biochemical theories of aging, Orgel's hypothesis[1] of "error catastrophe" is the most experimentally testable. Briefly, he suggested that a random error in a metabolic enzyme or process would be diluted out and not amplified, whereas an error in an enzyme or component involved in expression of genetic information would not only be passed on in time but also increase in magnitude by decreasing the fidelity of genetic expression. This process of error amplification would result in what Orgel termed "error catastrophe." Some possible points to look at in the expression of genetic information include transfer RNA, amino acid activating enzymes, enzymes involved in modifying transfer RNA, RNA polymerase, and others.

We have begun to investigate the spectrum of isoaccepting tRNA's by RPC-2 column chromatography and the amino acid activating enzymes in the $BC3F_1$ mouse strain. These mice live as long as three years, equivalent to more than 100 years for man. The comparison of tRNA profiles and enzyme activities in liver and brain between old and young animals should subject Orgel's hypothesis to an experimental test. Preliminary results on extent of charging with liver tRNA indicate no differences for serine, phenylalanine, tyrosine, aspartic acid, histidine, and arginine. These results were obtained using maximal charging conditions, determined separately for each amino acid.

Reference

[1] L. E. Orgel, *Proc. Natl. Acad. Sci. U.S.* **49**, 517–21 (1963).

1.31 A NEW ENZYME, ADENINE:AMP PHOSPHORIBOSYLTRANSFERASE, FROM *ARTEMIA SALINA*

F. J. Finamore Rose P. Feldman

The brine shrimp, *Artemia salina,* is incapable of synthesizing purines de novo and, as a consequence, converts guanine-containing nucleotides, principally P^1,P^4-diguanosine tetraphosphate, to adenine derivatives. In our studies of the mechanism by which this conversion occurs, we observed that the purine and ribose moieties of newly synthesized adenine nucleotides are each derived from different sources. Thus adenine nucleotides no not arise from a simple phosphorolytic cleavage of diguanosine tetraphosphate with a concomitant transformation of guanine to adenine; rather, our data suggest that diguanosine tetraphosphate serves as a source of adenine only and that another nucleotide or sugar phosphate donates ribose for the synthesis of adenine nucleotides.

Recently we succeeded in partially purifying an enzyme system from nauplii of *Artemia* that is capable of converting adenine to adenosine 5′-monophosphate without utilizing 5-phosphoribosyl-1-pyrophosphate as a source of ribose phosphate. In addition to adenine, the enzyme system has absolute requirements for PP_i, Mg^{2+}, and a cosubstrate which we have recently identified as adenosine 5′-monophosphate itself. Although in crude enzyme preparations other adenine-containing nucleotides, for example, ADP, ATP, NAD^+, NADH, and Ap_2A, can substitute for AMP, we believe they are active simply because they are first hydrolyzed to AMP by a phosphodiesterase that contaminates the preparations. Compounds that cannot yield AMP by action of the phosphodiesterase, for example, $NADP^+$, NADPH, cyclic AMP, S-adenosylmethionine, and α,β-methylene-ATP, are all inactive in the system.

We have been able to demonstrate that in the reaction free adenine is actually exchanged with the adenine moiety of added AMP. Thus we visualize the reaction as follows:

$$A^* + AMP + PP_i \overset{Mg^{2+}}{\rightleftharpoons} A^*MP + A + PP_i .$$

Inorganic pyrophosphate is absolutely essential for this exchange of adenine. Although its role in the reaction remains to be elucidated, it is clear that pyrophosphate does not contribute phosphorus to the product.

Since *Artemia salina* cannot synthesize purines de novo, we believe this enzyme functions by actively transporting free adenine within the organism itself or from the medium to the internal milieu of the organism for subsequent utilization.

1.32 THE PROMINENCE OF PHOSPHOCHOLINE TRANSFERASE IN CDP-CHOLINE SYNTHESIS IN *ARTEMIA SALINA*

R. D. Ewing

Extracts of nauplii of *Artemia salina* contain a microsomal enzyme which converts CMP to a compound identified as cytidine diphosphocholine. The reaction is completely dependent upon Mg^{2+} and/or Mn^{2+} ions and requires no other cofactors. If the phospholipids are labeled in vivo with either ^{32}P as orthophosphate or choline-1,2-^{14}C, radioactivity is incorporated in vitro into CDP-choline. In the presence of Mg^{2+} ions, the amount of ^{32}P and ^{14}C-choline found in CDP-choline is stoichiometrically equivalent to the amount of ^{32}P and ^{14}C-choline lost from phosphatidylcholine. In the presence of Mn^{2+}, however, ^{32}P is incorporated stoichiometrically in vitro from phosphatidylcholine to CDP-choline, but choline-1,2-^{14}C is not. Three possibilities would explain these results: (1) choline is exchanged at the CDP-choline level, (2) choline is exchanged at the phosphatidylcholine level, or (3) the source of the choline for CDP-choline formation is not phosphatidylcholine. We have found no evidence of choline exchange at the CDP-choline level, and we consider the third alternative to be unlikely. We are presently examining the possibility of choline exchange in phosphatidylcholine.

The reaction involving formation of CDP-choline is readily reversible, in that CDP-choline-1,2-^{14}C can be incorporated in the presence of Mg^{2+} or Mn^{2+} ions into a lipid identified as phosphatidylcholine by two-dimensional thin-layer chromatography. The equilibrium for this reaction, however, is strongly in the direction of CDP-choline synthesis. To test whether this enzyme is comparable with that described by Weiss, Smith, and Kennedy in rat liver,[1] we prepared a microsomal preparation from rat liver and assayed for the forward and backward reactions of the phosphocholine transferase. We found that the equilibrium for this reaction in rat liver microsomes also favors CDP-choline formation, although this effect is obscured at longer incubation times due to the presence of phosphatases in the preparation.

We conclude that the enzyme under investigation is CDP-choline-1,2-diglyceride phosphocholine transferase (EC 2.7.8.2). The equilibrium, however, is not in the direction of phosphatidylcholine synthesis, as previously described, but rather in the direction of CDP-choline formation. The reaction would presumably be of great importance during the synthesis and reorganization of membranous structures, since energy invested in the phosphatidylcholine molecule could easily be salvaged in the form of the high-energy intermediate CDP-choline.

Reference

[1] S. B. Weiss, S. W. Smith, and E. P. Kennedy, *J. Biol. Chem.* **231**, 53 (1958).

1.33 DISPROPORTIONATE INCORPORATION OF PURINE AND RIBOSE FROM UL-^{14}C-GUANOSINE INTO RNA AND DNA OF *ARTEMIA SALINA*

A. L. Golub

Larvae of the brine shrimp, *Artemia salina*, appear to have a complex nucleotide metabolism involving an extensive conversion of guanine-containing ribonucleotides into deoxyadenine nucleotides prior to the synthesis of DNA. Experiments performed in this laboratory[1] have suggested that purine and sugar may become separated during the interconversion and recombined prior to incorporation into DNA. Thus it was of interest to follow the in vivo distribution of base and ribose in larvae exposed to an exogenous source of uniformly labeled ^{14}C-guanosine.

UL-^{14}C-guanosine (base:ribose = 1:1) was given to larvae (36 hr) for additional incubation periods of 4, 24, 48, and 72 hr of development. Then acid-soluble nucleotides, RNA, and DNA were isolated from each

population. The base:ribose ratios were determined for the principal purine species following column chromatography. The results may be summarized as follows.

The purine:ribose ratio of dAMP from DNA remains 1:1 throughout the 72-hr incubation period, while that of dGMP from DNA is approximately 8:1 (purine:ribose) by 48 hr. An 8:1 ratio is also obtained for GMP from RNA after 48 hr. The ratio of AMP from RNA, however, shows a decline from 1:1 to approximately 0.3:1 during the same 48-hr period. These values are not immediately reflected in the acid-soluble nucleotides, but by 72 hr the acid-soluble adenine compounds show a reduced ratio of approximately 0.3:1, while that of the guanine compounds ranges from 5:1 to 8:1.

Thus it appears that unlabeled ribose is exchanging with the ribose of the UL-guanosine precursor for the production of GMP from RNA and DNA and that a source of unlabeled purine is being combined with labeled ribose for the production of AMP from RNA. The fact that AMP from DNA continues to have a 1:1 ratio throughout the 72-hr period suggests that UL-precursor is incorporated into DNA without extensive exchange with an unlabeled purine or ribose pool.

These results further suggest that AMP from RNA and dAMP from DNA are derived from different precursor pools. Since the ratio changes appear in the polynucleotides several hours before similar changes are detected in the acid-soluble pool, it may be that base or ribose substitutions taking place occur at the time the precursor is incorporated into the macromolecules, either at the enzyme itself or in a small pool immediately available to the enzyme. Later expression of these substitutions in the acid-soluble pool is probably due to degradation or turnover of the macromolecules at that time.

Further examination of the in vitro incorporation of nucleotides in the enzyme systems involved with the synthesis of RNA and DNA in these larvae is currently in progress.

Reference

[1] F. J. Finamore, unpublished results.

1.34 STIMULATION OF RNA SYNTHESIS IN STATIONARY LENS AND HUMAN SKIN CELLS

John Papaconstantinou W. A. Elmer[1]
Emilia M. Julku

During the growth of the vertebrate lens, the mitotic index of the epithelial cells in young embryos is high; it gradually decreases as the size of the lens approaches its mature adult size. Although these epithelial cells never lose the ability to divide, in the adult one rarely sees mitotic figures unless the tissue is injured. Thus in the adult vertebrate lens the epithelial cells are in an extended stationary phase. This is achieved by an indefinite prolongation of the G_1 phase of the cell cycle.

Our previous studies have shown that very significant regulations of RNA and protein synthesis occur in stationary lens epithelial cells. Both RNA and protein syntheses are greatly reduced. More detailed analyses have shown that mRNA is stable in these cells, that ribosomal RNA synthesis is greatly reduced, and that only approximately 50% of this RNA is assembled into mature ribosomal subunits.

When adult lenses are organ cultured, the epithelial cells are stimulated to reenter the cell cycle. At this time both mRNA and rRNA syntheses rise to the level seen in rapidly dividing cells of the fetal lens. The mRNA is no longer stable, and rRNA is completely processed into 28s and 18s forms. These observations indicate that "repressor molecules" in vertebrate cells may exist in high concentrations in stationary cells. Thus studies on the nature of these molecules might be facilitated by using a system in which cells can remain in a prolonged stationary phase and can be stimulated to reenter the cell cycle at will. The intact adult lens meets these requirements, as do many cells in tissue culture which exhibit contact inhibition.

We initiated our studies by assuming (1) that these repressor molecules may be proteins, (2) they are synthesized during the stationary (extended G_1) phase, and (3) inhibition of protein synthesis in stationary cells should result in a stimulation of RNA synthesis. To test this hypothesis, cycloheximide was used to inhibit protein synthesis in (1) adult lens epithelial cells, (2) contact-inhibited lens epithelial cells and human skin cells in tissue culture, and (3) epithelial cells from organ-cultured lenses. In the presence of cycloheximide, RNA synthesis was stimulated in non-organ-cultured lens epithelial cells and in both types of contact-inhibited cells in culture. RNA synthesis was inhibited in the organ-cultured lens epithelial cells. (This agrees with observations from other laboratories that cycloheximide inhibits RNA synthesis in HeLa cells and other rapidly dividing cells.)

Analyses were done to determine whether specific species of RNA are selectively stimulated by cycloheximide. RNA was simultaneously labeled with [$^{14}CH_3$] methionine and [^{3}H] uridine and fractionated on MAK columns or by sucrose density

gradient centrifugation. MAK fractionation shows that cycloheximide treatment does not stimulate rRNA synthesis, as indicated by RNA methylation, whereas uridine incorporation into fractions eluted by high salts in the post-rRNA regions of the column are greatly stimulated. These data indicate that DNA-like RNA is selectively stimulated by cycloheximide treatment. Sucrose gradient analyses show that the RNA stimulated is heterodisperse, although there is an indication that the nonmethylated high-molecular-weight RNA (>28s) shows the greatest degree of stimulation. In addition, these sucrose gradients showed no increase in the methylation of 28s and 18s RNA.

In conclusion, our experiments at this stage of the project indicate that cycloheximide selectively stimulates mRNA synthesis only when cells are in a stationary phase. Further experiments are being done to define the mechanism of this specific stimulation of RNA synthesis.

Reference

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1.35 AGING OF α-CRYSTALLINS DURING DEVELOPMENT OF THE LENS

Winifred G. Palmer[1] John Papaconstantinou

α-Crystallin is a structural protein present in large quantities in the vertebrate lens. The bovine protein has a molecular weight of about 1×10^6 and is composed of subunits whose molecular weights are approximately 25,000. α-Crystallin can be disaggregated into its component subunits by 7 *M* urea + 3×10^{-3} *M* EtSH. Fractionation of the subunits is achieved by chromatography on urea-equilibrated DEAE-cellulose columns in the presence of EtSH. Under these conditions four subunit fractions can be resolved from the urea-treated adult protein. Each subunit has a unique pattern on gel electrophoresis. Fetal α-crystallins have also been fractionated on urea-equilibrated DEAE columns and were found to contain three of the four subunits found in the adult protein. This missing subunit can also be detected in α-crystallins from calf lenses, but not in as high a concentration as seen in the proteins from adult lenses. Further studies on the biochemical and immunochemical properties of this subunit indicate that it is gradually added to the fetal protein during the growth of the lens, not as a result of de novo protein synthesis but rather as a result of the conversion of one subunit (II_a) to another (II_b). Our conclusions are based on the following experimental observations: (1) Subunits II_a (fetal + adult) and II_b (adult) are immunochemically identical by the Ouchterlony double diffusion test. (2) In the fetal lens radioactive amino acids are incorporated into all three subunits (I_a, I_b, and II_a). In both calf and adult lenses there is no incorporation into subunit II_b, although the other subunits are significantly labeled. This indicates that the formation of subunit II_b does not occur by de novo protein synthesis. (3) Subunits II_a and II_b have the same amino acid composition and the same molecular weight (25,000). This eliminates the possibility that II_b may be a dimer of II_a. (4) Subunits II_a and II_b have unique chromatographic and electrophoretic properties. On the basis of these observations, we conclude that subunit II_b is not a product of gene activation but is gradually formed by the conversion of subunit II_a to II_b by a posttranslational reaction. The electrophoretic and chromatographic properties indicate that this conversion results in an increase in the net negative charge of the protein. We believe that this conversion may involve a chemical alteration of a specific amino acid side group, and that the reaction which brings about this change may be associated with the phenomenon of cellular aging.

Reference

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1.36 STUDIES ON THE RATE OF SYNTHESIS AND DEGRADATION OF α-CRYSTALLIN IN LENS CELLS DURING DIFFERENTIATION AND AGING

Jean Delcour John Papaconstantinou

We have shown in our previous work that stabilization of mRNA templates in the lens is associated with fiber cell maturation and with the age-related slowing down of mitotic activity of the adult lens epithelial cells. Both cellular maturation and the regulation of mitotic activity are important events occurring in cellular differentiation. In both cases the stabilization of mRNA is accompanied by the slowing down of protein synthesis. Since protein synthesis is affected, we initiated this project to determine the rate of synthesis and rate of degradation of lens proteins under conditions where the mRNA is rapidly turning over (dividing lens epithelial cells) and under conditions where mRNA is stabilized (fiber cells and stationary epithelial cells of

the adult lens). The problem is being approached in the following way: The rate of synthesis and degradation of the total soluble protein fraction will be determined. This will be followed by a determination of the rate of synthesis and degradation of the native α-crystallin molecule (mol. wt. 1×10^6) and of the four α-crystallin subunits (mol. wt. 25,000).

To facilitate these studies two methods have been devised for a rapid and efficient purification of the native α-crystallin molecule: (1) Chromatography on DEAE-cellulose using 0.02 *M* phosphate buffer, pH 5.7. Under these conditions the β- and γ-crystallins are eliminated from the column by continued elution with the same buffer, whereas the α-crystallins remain adsorbed. The α-crystallins are then eluted from the column with 0.1 *M* phosphate buffer, pH 5.7, plus 0.2 *M* NaCl. This procedure has been shown to produce immunochemically and electrophoretically pure α-crystallin (Palmer and Papaconstantinou). (2) Native α-crystallins have an $s_{20,w}$ ranging from 18 to 21. By sucrose density gradient (5–20%) centrifugation a very distinct, pure fraction can be prepared from homogenates. The β-crystallins (mol. wt. 60,000) and γ-crystallins (mol. wt. 25,000) remain at the top of the tube. This rapid method facilitates the use of small quantities of material and a simultaneous analysis of several samples.

The theoretical and experimental conditions for determination of rate of synthesis and degradation of proteins in the excised intact lens have been established. The rate of synthesis will be determined under conditions in which the amino acid composition of the incubation medium approximates that of the vitreous humor; the amino acids are not a rate-limiting factor, and the specific activity of the radioactive amino acid in the pool is experimentally determined. The rate of degradation will be determined under conditions in which the radioactive amino acid in the pool is rapidly eliminated and the loss of incorporated amino acid can be determined directly. These experiments have been done, and the experimental conditions described above appear to fulfill the requirements for these studies. The following incubation system has been established: Intact lenses are incubated in a modified Eagle's MEM medium in which the total amino acid concentration approximates that of the vitreous humor. ^{3}H-Leucine is added in concentrations ranging from 10 to 25 μc/ml. To measure the rate of synthesis, lenses are removed from the medium at successive time intervals. Amino acid analyses of the TCA-soluble fraction will be done to determine the pool size and the ratio of radioactive leucine to total leucine concentration in the pool. The rate of protein synthesis will be determined after the ^{3}H-leucine in the pool reaches a constant level. At this time the rate of protein synthesis is constant. The TCA-insoluble fraction will also be determined. To determine the rate of degradation, lenses are incubated in the presence of ^{3}H-leucine for 2½ hr. The radioactive medium is replaced several times by non-isotope-containing medium. This results in a rapid loss of radioactive amino acid from the pool, and the loss of incorporated amino acids will be determined when the concentration of radioactive amino acid in the pool approaches zero. Determination of the total amino acid pool will also be done in these experiments to show whether any of the leucine has been converted to other amino acids.

Preliminary results using these procedures show that the rate of synthesis of total soluble proteins is identical for the cortex fiber cells of young and old adult lenses. Since fiber cell formation at both ages results in mRNA stabilization, these data indicate that there is no age-related change in the rate of total protein synthesis in cells having stable mRNA templates. Experiments are now being carried out to measure the rate of synthesis and degradation of total soluble protein in epithelial and cortex fiber cells to determine whether the stabilization of mRNA will affect these parameters. In addition the experiments on the native α-crystallin molecule have been started.

1.37 A STUDY ON THE REGULATION OF RNA SYNTHESIS DURING EMBRYONIC INDUCTION

E. F. DuBrul John Papaconstantinou

It is now widely accepted that specific stages of embryonic development are associated with qualitative and quantitative changes in RNA synthesis. The qualitative changes, which are believed to be due to gene activation, have been shown mainly through DNA-RNA hybridization studies in amphibian and sea urchin embryos and in regenerating liver. Very little is known about the changes in RNA synthesis which accompany the process of cell-cell interactions during embryonic induction. These cellular interactions are the developmental events in embryogenesis which result in the initiation of specific pathways of cellular differentiation. It is our purpose in these studies to describe the qualitative changes of RNA species which may occur as a result of gene activation during embryonic induction. The long-term goal of this project is to gain an understanding of the mechanism by which cellular interactions result in the determination of a specific

pathway of cellular differentiation. The system we have chosen to study is the induction of feather formation in the chick. In this system the interacting cells are the dermis (mesodermal in origin) and epidermis (ectodermal in origin). This interaction results in the initiation of tissue-specific proteins, the keratins, which are utilized for the formation of a specific structure, the feather.

Our experimental approach to this project is as follows: (1) A characterization of the avian genome into repeating and unique sequences of DNA. Radioactive chick DNA has been prepared from embryonic liver and brain. Both native and denatured DNA have been characterized by CsCl equilibrium density gradient centrifugation and have been successfully fractionated on hydroxyapatite columns. We are presently determining the percentage of the total genome that makes up repeating sequences and unique sequences. Separate DNA-RNA hybridization studies will be done using these repeating and unique sequences to determine whether gene activation during induction involves the transcription of these specific regions of the genome. (2) Attempts will also be made to determine the type of RNA synthesized during induction. By acrylamide gel electrophoresis the size of the RNA's synthesized can be determined. Nuclear and cytoplasmic fractions will also be analyzed in this series of experiments to determine the cellular localization of new RNA species. These will also be analyzed by DNA-RNA hybridizations to determine their genomic origin.

In general, the information derived from these experiments should give an indication of the RNA species involved in translation of new proteins synthesized (cytoplasmic RNA) and RNA species which may be involved in a regulatory role (nuclear RNA).

1.38 SPECIFIC SYNTHESIS OF A UNIQUE DNA DURING DEVELOPMENT OF *RHYNCHOSCIARA* SP. TESTIS

E. T. Chin[1] W. S. Bradshaw
John Papaconstantinou

The larvae of *Rhynchosciara* sp. develop synchronously at the organismic cellular and molecular levels. During the latter half of fourth instar development, DNA synthesis occurs both in somatic cells and germ cells, even though these cells do not undergo mitosis. CsCl density gradient analysis of the DNA synthesized in the testis, salivary gland, fat body, and intestine has revealed the synthesis of a unique DNA found only in the testis. This unique testis DNA has a density of 1.679 g/cm^3 based on a density of 1.710 g/cm^3 for *E. coli* DNA. On the basis of this density, the testis DNA has a G + C content of 25%. The DNA peak common to both testis and salivary gland has a density of 1.694 g/cm^3 and a G + C content of 33%. An analysis of the density change in ethidium bromide–treated testis DNA showed a change of 0.070 g/cm^3 for the satellite DNA and 0.124 g/cm^3 for the main-band DNA. This significantly smaller change in the density of the satellite DNA strongly indicates that it has a circular structure.

A temporal analysis of DNA synthesis in the testis during fourth instar development has shown that the rate of incorporation of [^{3}H] thymidine into the unique DNA is equal to or greater than that of the heavier DNA during a very limited period centered around the 36th day after hatching. Thus the synthesis of this low-density DNA appears to be specific for both the tissue and the period of development. During this period of development, the spermatogonia undergo dramatic cytological changes involving an increase in cell size and a dramatic enlargement of the mitochondria. The low G + C content and circularity of this DNA indicate that it may be associated with the mitochondria. Furthermore, the sharp increase in synthesis of this DNA may be associated with the structural changes the mitochondria undergo at this stage of development. Experiments are under way to determine the cellular location of this unique DNA.

Reference

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1.39 DIFFERENTIAL SYNTHESIS OF SALIVARY GLAND DNA DURING FOURTH INSTAR DEVELOPMENT OF *RHYNCHOSCIARA* SP.

W. S. Bradshaw John Papaconstantinou

Late in the fourth larval instar the polytene chromosomes of the salivary glands of *Rhynchosciara* undergo changes in morphology characterized by a pattern of band-specific puffs. Autoradiographic analysis utilizing 5-bromodeoxyuridine (BUdR) have demonstrated a dramatic increase in grain density due to differential incorporation of this DNA precursor analog into several of these bands. The temporal sequence of differential DNA synthesis and its relationship to puff activity at these loci have been established. In view of the significantly greater rate of synthesis of DNA in the

puff regions, BUdR was used to make this DNA denser to facilitate its separation from the rest of the DNA by CsCl gradients. Bulk preparations of this BUdR-labeled DNA have been subjected to CsCl equilibrium density gradient centrifugation. The profile of these gradients includes a region of heterodisperse DNA molecules having densities in the range of 1.70 to 1.72 g/cm^3 in addition to a peak at a density of 1.695 g/cm^3. The single peak having the latter density characterizes [^{3}H] thymidine-labeled DNA from larvae of the same age. We interpret these data to indicate that the heavy, heterodisperse DNA may represent rapidly synthesized DNA of the puff regions, whereas the lighter DNA peak represents the less rapidly synthesized DNA which did not incorporate enough BUdR to bring about a change in density. Identification of the high-density fraction as puff-specific DNA is presently being done by hybridization with chromosomal DNA in cytological preparations. Hybridization studies are also being carried out to determine whether DNA puffing is a result of gene amplification. These studies should provide insight into the phenomenon of gene amplification as a regulatory mechanism in development.

1.40 EFFECT OF ALLOGENEIC AND XENOGENEIC BONE MARROW ON THE 30-DAY LD 50 OF X-IRRADIATED MICE

D. G. Doherty Frances E. Hacker

Our previous studies with syngeneic marrow established a triphasic response in the LD 50 of x-irradiated mice treated with a graded series of nucleated cell doses. The response was found to be most efficient over slightly less than a decade of cell dose, and, in addition, very large cell doses provided some protection against intestinal damage.[1] This study has been extended to allogeneic and xenogeneic marrow to determine whether a similar response to cell dose could be obtained, whether there was an optimum cell dose for maximum recovery, and the incidence of chimerism in the surviving mice. Female (C57BL/Cum ♀ X C3H/Anf Cum ♂) mice were exposed to various doses of x radiation and on day 1 injected intravenously with a nucleated cell dose of either BALB/c Cum ♀ X DBA/2 Cum ♂ mouse marrow or Fisher 344 rat marrow. The surviving animals were tested for chimerism at 90 days after irradiation. The survival results are presented in Fig. 1.40.1, with the syngeneic cell curve included for comparison purposes. The allogeneic marrow can be seen to give a triphasic response similar to the syngeneic marrow, although the LD 50 values at comparable cell doses are 200 to 300 r lower. The xenogeneic marrow, as might be expected, was ineffective except at the relatively high 50 X 10^6 cell dose level, which provided good 30-day survival up to 1100 r but enabled few animals receiving more than 850 r to survive past 90 days. Although there have been reports of a 710-r death in previous foreign marrow experiments, no such death pattern was observed in either the mouse or rat marrow experiments at all cell doses and radiation doses between 500 and 900 r. The pattern of chimerism in the allogeneic experiments was complex, depending on both the cell and radiation dose. At cell doses of 1 X 10^7 and below, the red cell type was that of the host up to 700 r. Between 700 and 900 r the types were divided between host and donor, while above 900 r all were of donor type. Higher cell doses produced a greater percentage of chimeras at the lower radiation doses. In one experiment where AET pretreatment was combined with allogeneic marrow, this pattern of chimerism was shifted to higher radiation doses, indicating some protection to the immune system. The chimerism results with the xenogeneic marrow were comparable with the allogeneic, although fewer chimeras were obtained.

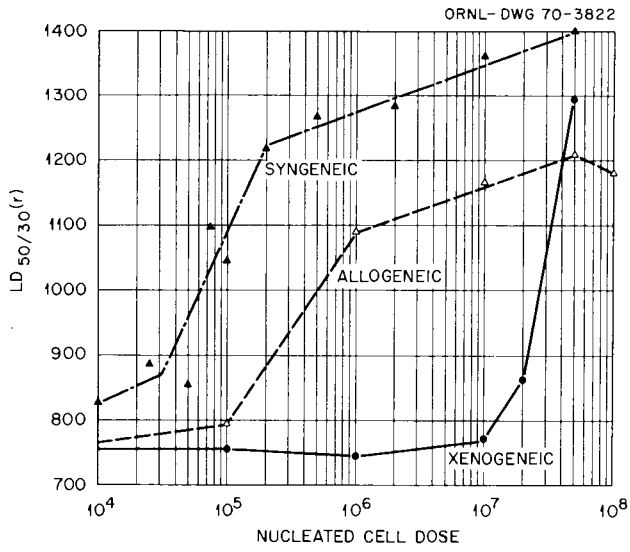

Fig. 1.40.1. $LD_{50/30}$ as a Function of Bone Marrow Cell Dose.

Reference

[1] D. G. Doherty, *Radiation Res.* **37**, 299 (1969); D. G. Doherty and L. H. Smith, *Radiation Res.* **40**, 85 (1969).

1.41 IN VITRO CHEMICAL PROTECTION OF X-IRRADIATED MOUSE BONE MARROW CELLS

D. G. Doherty L. H. Smith

Evaluation of chemical radioprotectors has relied primarily upon their assay in the lethally irradiated mouse. The complexity of this system makes it difficult to interpret structure and activity studies in mechanistic terms. In addition, toxicity of many compounds which potentially may be good radical-trapping or repair agents precludes their examination in the whole animal. Although evaluation of the effectiveness of radioprotectors must, in the final analysis, be made in intact animals, there are practical and theoretical advantages for studying chemical protection in isolated mammalian systems. We have therefore turned to development of a simpler system with less stringent toxicity requirements and free from the complexity of overall animal metabolism. The spleen-colony-forming assay of Till and McCulloch[1] seems well suited to this purpose, since the survival of hemopoietic stem cells has long been recognized as essential to recovery of mice exposed to sublethal to supralethal radiation. Quantitative correlation has been obtained from both the survival curves in exogenous marrow transplantation studies and from the colony-forming assay. One of us[2] showed that marrow cells irradiated in vitro in the presence of protective compounds and injected into lethally irradiated mice increased their survival.

To utilize advantages of an isolated system, we began a study of chemical protection of mouse bone marrow cells irradiated in vitro and then tested in vivo for reproductive capacity using the spleen-colony-forming method. The technique consists in suspending marrow cells in fetal calf serum at a cell concentration of 5×10^6/ml. Portions of the suspension are placed in plastic dishes to which the desired chemical is added. After a short incubation, suspensions are exposed to ionizing radiation, then diluted to a concentration of 0.150×10^6 cells/ml. This number of cells is injected into syngeneic recipients that have been exposed to 850 r of 250-kvp x rays. Ten days later, mice are killed, spleens removed and fixed, and the number of spleen colonies counted. Using the number of spleen colonies produced by 0.150×10^6 unirradiated cells as 100% survival, cell inactivation curves can be constructed which relate survival to in vitro x-ray exposure (90, 180, 270, 360 r).

Our initial experiments were designed to define the in vitro system. In terms of spleen-colony-forming capacity of bone marrow, cell inactivation curves yield a D_{37} of 115 r and an n of 1.6. AET was used for the initial studies, and it was found that the D_{37} is related directly to AET concentration and to incubation temperature (0–37°C, 15 min) with AET before irradiation. Other chemicals are being tested, including certain aminoalkylphosphorothioates which protect the mouse but do not protect in the in vitro system. These compounds, therefore, must undergo structural alteration in vivo to their protective form.

Extension of the in vitro system promises several advantages, especially when applied to the measurement of the protection obtained with radiation of a different RBE.

References

[1] Till and McCulloch, *Radiation Res.* **14**, 213 (1961).
[2] *Exptl. Cell Res.* **13**, 627 (1957).

1.42 STUDIES ON THE SUBSTRATE SPECIFICITY OF PEPSIN

D. G. Doherty Margaret A. Turner Jesse James

Our previous studies on the pepsin hydrolysis of acylated dipeptide ester substrates have been principally concerned with the effects of alterations in the peptide side chain and acyl group on the kinetic constants in order to determine the main specificity requirements for pepsin action. In the course of this work it was found that due to limited solubilities and low Michaelis constants, the serial sampling colorimetric method that had been developed was not sufficiently precise to yield reliable constants for some of the modified substrates. We consequently developed a continuous-sampling sensitive procedure based on the Technicon amino acid analysis system for the kinetic analyses. The results are punched out on IBM cards and the kinetic constants calculated by a computer program. Using this new procedure we are examining modifications of the C terminal end of various substrates. Conversion of the nicotinoyl- or isonicotinoylphenylalanoylphenylalanine ethyl esters to the free acids raised the K_m by a factor of nearly 8 and decreased the reaction rate markedly. Similar results were obtained with an uncharged, for example, chloroacetyl, acyl group, the K_m for the free carboxyl group being 3.3×10^{-4} compared with 0.5×10^{-4} for the ester. The bromoacetyl dipeptide had a higher K_m of 5.4×10^{-4}, indicating that acyl group size was a factor on the N terminal end. The structure of both the chloroacetyl and bromoacetyl dipeptide esters has been determined by x-ray crystallographic analysis by C. H. Wei and R. Einstein, and the configuration is in agreement with the requirements postulated from kinetic studies. Since pepsin seems to have a different specificity where proteins are concerned, we are preparing a series of large polypeptides to examine these differences by kinetic method.

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2.1 EFFECT OF SUBZERO TEMPERATURES DURING THE FREEZING AND THAWING OF CHINESE HAMSTER TISSUE CULTURE CELLS

Peter Mazur S. P. Leibo E. H. Y. Chu

Two factors are responsible for the death of microorganisms during freezing: intracellular ice formation and changes in the properties of the suspending solution such as the concentration of solutes. We have recently demonstrated that these two factors also can explain the death of frozen and thawed marrow stem cells and hamster tissue culture cells.[1,2] The purpose of still more recent experiments was to study the temperatures at which the latter are killed during cooling and during warming and to determine the effect of the suspending medium on those temperatures. We had shown that hamster cells in glycerol, dimethyl sulfoxide (DMSO), sucrose, and polyvinylpyrrolidone (PVP) exhibit maximum survival at some optimum rate of cooling to −196°C, the value of which depended on the specific additive and its concentration.[1] We have now shown that cells suspended in all four additives are much more sensitive to slow warming when they have previously been frozen at rates faster than optimum than when frozen at rates slower than optimum. Moreover, the rate of inactivation of cells cooled to and held at a given temperature is higher following rapid cooling than following slow cooling and is higher at higher subzero temperatures. For example, cells suspended in 0.4 *M* glycerol and cooled at 300°C/min to −40°C or below are almost totally inactivated after 15 min at −40°C. We believe all these results indicate that injury results from the recrystallization of intracellular ice formed during cooling at higher rates. This hypothesis is to be tested by an electron microscopic examination of freeze-etched cells.

The temperature at which inactivation occurs during the slow warming of rapidly cooled cells bears little relation, however, to the temperature of inactivation during the slow cooling of the cells in the same additive (Table 2.1.1). The most widely accepted current hypothesis is that damage during slow cooling results from the concentration of electrolytes and that equal molarities of additive should produce an equal decrease in both the total amount of inactivation and in the temperature at which it occurs. The data in column 2 are clearly inconsistent with the latter. Previous data were inconsistent with the former.[1]

Table 2.1.1. Inactivation Temperatures of Chinese Hamster Cells During Slow Cooling and Slow Warming

Suspending Medium	Median Lethal Temperature During −	
	Slow Cooling, 1.6°C/min	Slow Warming, 1°C/min
Hanks' balanced salt solution (HBSS)	−8	
0.4 *M* sucrose + HBSS	−15	−50
0.4 *M* glycerol + HBSS	−22	−32
0.004 *M* PVP + HBSS	−30	−45
0.4 *M* DMSO + HBSS	<−60	−18

References

[1] P. Mazur *et al.*, *Cryobiology* **6**, 1–9 (1969).
[2] S. P. Leibo *et al.*, *Cryobiology* (in press).

2.2 OSMOTIC DAMAGE TO HUMAN AND BOVINE ERYTHROCYTES

E. M. Ross[1] S. P. Leibo Peter Mazur

The most generally accepted explanation of hemolysis caused by freezing and thawing of red blood cells ascribes much of the damage to osmotic effects resulting from the concentration of solutes due to the removal of water as ice and to subsequent dilution of the solute upon thawing. This explanation states that when a cell is frozen in solutions of normally nonpermeating solutes such as NaCl or sucrose, the permeability of the membrane is altered by exposure to the concentrated solute. This damage is manifested as hemolysis upon dilution during thawing. Our data are consistent with the view that the damage to the membrane is physical rather than biochemical in origin, since the behavior of the cell in sucrose is identical to that in NaCl. Our data show that there are two distinct hemolytic events. The first occurs when solute concentration is increased above 1.5 osmolal and is a function only of the initial concentration to which the cell is exposed. The second, referred to above, occurs when the cell is first exposed to concentrations ≥2.0 osmolal and then is diluted back to concentrations ≤1.2 osmolal. Dilution back to concentrations greater than this second value does not produce hemolysis regardless of the initial concentration. A computer simulation of these events is being developed for comparison with experimental data.

The effect of permeating solutes was investigated with glycerol solutions. With isotonic saline in the medium to prevent immediate hemolysis, red cells may be exposed to high concentrations of glycerol, with hemolysis occurring *if* and only *if* they are subsequently diluted by at least a factor of 2. Apparently, after

exposure to the concentrated glycerol, enough glycerol permeates the cell so that, upon dilution, water enters the cell, increasing its volume to the point at which it ruptures. This explanation is also supported by the following facts:

1. Bovine red cells, which are less permeable to glycerol than are human cells, require longer exposure to glycerol before hemolyzing upon dilution.
2. Human red cells in the presence of 3×10^{-5} *M* Cu^{2+}, which reduces the permeability of the cell to glycerol, also require longer exposure before hemolyzing upon dilution.
3. Cells exposed to glycerol at lower temperatures also require longer exposure before hemolyzing upon dilution.
4. Slow dilution of cells in glycerol prevents hemolysis by allowing time for glycerol to diffuse out of the cell.

Furthermore, by applying classical differential equations for membrane permeation to the above explanation, we have achieved computer simulation of these phenomena.

Reference

[1] Student trainee.

2.3 EFFECT OF MACROMOLECULES ON THE ABILITY OF WATER TO SUPERCOOL

Peter Mazur R. H. Miller

A number of investigators have speculated that the hydration layer around macromolecules and much of the water in cells is "icelike." One measure of the extent to which it is truly icelike would be the extent to which it can reduce the ability of water to supercool. Small samples (0.02 ml) of deionized, glass-distilled water in glass tubes were capable of being supercooled to −20 to −22°C, provided that the water was Millipore-filtered, the glassware carefully washed in filtered water, and all manipulations carried out in a laminar flow hood. This ability to supercool was *not* affected by the following solutes: NaCl, citrate buffer, Tris buffer, native calf thymus DNA, denatured DNA, collagenase, pronase, or trypsin. Solutions of soluble calf-skin collagen, in contrast, only supercooled to −10 to −11°C. Such solutions are slightly opalescent. When they were centrifuged at 9000 *g* for 30 min, the nucleating ability was found to be located in the pellet rather than in the supernatant soluble collagen. The ability of the pellet to nucleate is heat labile (10 min at 80 to 90°C destroys the activity) and nondialyzable. It is not affected by collagenase or trypsin, but it is destroyed by pronase. The protein in the pellet is less than 10% of the total protein in the collagen solutions as measured by the Lowry technique and by vacuum drying on a Cahn balance. Since, with the exception of this pellet material, the water around macromolecules shows no ability to nucleate supercooled water, to this extent it fails to be icelike.

2.4 HISTONE-DNA INTERACTION

D. E. Olins Ada L. Olins

The present studies have continued to define the macromolecular structure of soluble reassociated histone-DNA complexes as models for native chromosomal nucleohistones.

As mentioned earlier[1,2] at least one class of histones, the lysine-rich (or f1) histones, appears to stabilize the structure of native DNA in the B form by binding within the wide groove of the double helix, neutralizing phosphate charges, and cross-linking the antiparallel strands. This same histone has been shown, by Langan and co-workers, to be phosphorylated at a single serine residue by a histone kinase whose activity increases with hormonal stimulation. Complexes of phosphorylated and nonphosphorylated f1 histones with native DNA have been compared by thermal denaturation and renaturation, absorption spectroscopy, and circular dichroism. No significant differences were observed. Thus, if histone phosphorylation is an important causal factor in chromosomal expression, conformational changes due to histone modification are probably highly localized, or absent, in solution. Careful circular dichroism studies have been extended down to 205 nm. Difference measurements indicate less than 30% α helix within the f1-DNA complexes.

Two additional histone classes, f2a1 and f2a2, have been isolated by gel filtration. These preparations exhibit greater than 90% purity by disk electrophoresis and amino acid analysis. Complexes with DNA have been made. Both f2a1 and f2a2 produce marked thermal stabilization of native DNA, although the melting characteristics are very different from f1-DNA complexes. Furthermore, circular dichroism measurements indicate that the DNA remains in the B form when covered with f2a1 or f2a2 histones. The complete amino acid sequence of f2a1 histone has been described by Bonner and co-workers and by Busch and co-workers. We have made space-filling molecular models

of native DNA and f2a1 histone and have examined possible ways of interaction between them and consequences to various protein functional groups.

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2.5 PREPARATIVE ISOLATION OF (G + C)-RICH SATELLITE DNA FROM THE LAND CRAB

Dale E. Graham[1]

The (G + C)-rich satellite DNA of the Bermuda land crab, *Gecarcinus lateralis*, comprises only 3% of the total DNA[2] and therefore may only laboriously be isolated by centrifugation in preparative CsCl gradients. However, a modification of thermal chromatography on hydroxyapatite permits the rapid isolation of this satellite DNA in native, uncontaminated form.

Sodium phosphate buffer (0.16 *M*) elutes DNA dissociated by increasing temperature from hydroxyapatite columns; undissociated DNA remains bound. At any given temperature undissociated DNA can be eluted by raising the phosphate concentration to 0.5 *M*. Since the temperature of DNA strand separation increases linearly with increasing (G + C) content, thermal chromatography can be used to separate DNA's of different base compositions.

However, because the DNA duplex is stabilized by both the phosphate buffer and the hydroxyapatite crystals, the temperature of elution of 47.5% (G + C)-containing DNA is 95°C,[3] the upper limit for thermal chromatography. In order to isolate DNA's of high (G + C) content, uncontaminated by DNA's dissociating at temperatures higher than 95°C, I have taken advantage of the observation that, in solution, 7.2 *M* $NaClO_4$ lowers the T_m of various DNA's by 45°C, as compared with its T_m in 0.1 *M* NaCl.[4] A buffer composed of 0.12 *M* sodium phosphate and 7.2 *M* $NaClO_4$ decreases thermal elution temperatures by more than 30°C.

This modification has allowed the isolation of native (G + C)-rich DNA species which would be difficult to isolate by other means of DNA fractionation. For example, the satellite and main-band DNA's of calf thymus are of such similar base composition as to be incompletely resolvable by analytical CsCl centrifugation; using the $NaClO_4$ modification, it is possible to enrich greatly for the (G + C)-rich satellite from calf thymus DNA.

References

[1] Student at the UT–Oak Ridge Graduate School of Biomedical Sciences.
[2] D. M. Skinner, *Proc. Natl. Acad. Sci. U.S.* **58**, 103 (1967).
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2.6 EFFECT OF LIMB LOSS AND MOLTING HORMONE INJECTIONS ON THE LAND CRAB, *GECARCINUS LATERALIS*

Dale E. Graham[1] Dorothy M. Skinner

In a random population of land crabs (*Gecarcinus lateralis*) maintained under standard laboratory conditions each animal molts approximately once per year. For our studies on growth and regeneration, we require a method for initiating precocious molts. We therefore injected several series of animals with graded doses of the arthropod molting hormone, ecdysterone. Since we had preliminary evidence that animals which were missing many walking legs or both claws molted more frequently than those which retained all their limbs, we removed either the walking legs or the claws from several series of animals (designated L^- animals). Some of these were also injected with the hormone. All the L^- animals regenerated their missing legs and molted after a mean elapsed time of 53 days. Injection of ecdysterone at 6, 12, or 18 μg/g did not further reduce the time to the next ecdysis. Injection of ecdysterone at the same levels into animals which retained their limbs did not cause precocious molting. Since the hormone preparations were effective in causing the expected chromosomal puffs when injected into *Rhyncosciara* (E. Mattingly, personal communication), we conclude that limb loss is a more effective stimulus to molting than the present preparations of ecdysterone. This procedure is therefore a very effective way of inducing the molting cycle for the purpose of studying development and regeneration in *G. lateralis*.

Reference

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

2.7 SUBCELLULAR LOCALIZATION OF DNA SATELLITES OF VARIOUS CRUSTACEAN SPECIES

Marilyn S. Kerr Wanda G. Beattie
Dorothy M. Skinner

Our first effort in determining the biological role of the two satellite DNA's in the Bermuda land crab, *Gecarcinus lateralis,*[1] has been to localize them within the cell. During some of these experiments in which we have enriched for various subcellular organelles, we have also isolated another species of DNA which is not detectable in DNA preparations from whole tissues. We found this satellite DNA in all four widely divergent species of Crustacea we studied: These were three members of the suborder Brachyura (*Gecarcinus,* a land crab; *Cancer borealis* and *Callinectes sapidus,* marine crabs) and one member of an entirely different suborder *Homarus americanus,* the lobster).

When either hemocytes or ovaries from specimens of *Gecarcinus* labeled with ^{14}C-thymidine are lysed and centrifuged to equilibrium in alkaline CsCl gradients, the DNA forms two distinct bands with densities characteristic of the main band and "poly d(A-T)" common to this animal. Since with minimal manipulation[2] the DNA separates into two distinct bands, these data are consistent with the concept that either (*a*) the bond joining the segments rich in d(A-T) to the remainder of the DNA is labile, (*b*) the two classes of DNA are physically separated in vivo but are located in the same organelle, or (*c*) they may even be located in different subcellular organelles. Therefore we devised methods for subcellular fractionation of crustacean tissues. For some of these experiments, species of Crustacea considerably larger than *Gecarcinus* were selected to enable us to obtain more tissue per animal. Homogenates of midgut gland (hepatopancreas) or ovary of various crustacean species were centrifuged on discontinuous sucrose gradients, and fractions were collected. Those isolated from the region characteristic of mitochondria were found to contain more than 95% of the particle-bound cytochrome oxidase activity and no acetocarmine-staining particles. These fractions were further purified by differential centrifugation and treatment with DNase. (The bacterial contamination in the various fractions is slight; our final mitochondrial fractions contain three orders of magnitude fewer bacteria than the number required to yield the amount of DNA we recover.) Electron microscopy of our mitochondrial preparations showed them to contain structures with the morphology typical of mitochondria.

On analytical centrifugation in CsCl, DNA isolated from highly purified mitochondrial fractions was found to be composed of a d(A,T)-rich DNA, ρ = 1.687 g/cm^3. In similar experiments the mitochondria from all four species yielded DNA of this density. The choice of homogenizing medium determined the types of DNA isolated from nuclei. Nuclei isolated in media containing high concentrations of Mg^{2+} yielded only a very broad peak of main-band DNA. When the Mg^{2+} was replaced by KCl and EDTA was added to inhibit nucleases, in addition to main-band DNA, the (G + C)-rich satellite DNA (ρ = 1.721 g/cm^3) and the "poly d(A-T)" satellite were found in nuclear fractions.

References

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2.8 ORIGIN OF CHROMOSOME REPLICATION IN *ESCHERICHIA COLI* K12

L. G. Caro Claire M. Berg[1] D. P. Allison

Attempts to define the origin of chromosome replication in *Escherichia coli* K12 by synchronizing replication with a cycle of amino acid starvation followed by reinitiation gave results that were difficult to interpret. A new approach was taken that made use of the fact that cells growing in rich medium may have several growing points per chromosome. Such cells would show a gradient in the frequency of markers, from origin to end, steeper than cells with a slower growth rate.

We analyzed, for a number of markers, the transducing activity in P1 lysates made on fast-growing cells and on slow-growing cells. The results established:

1. that fast-growing cells use premature initiation, resulting in multiforked replication, as a means of control for chromosome replication;
2. that the origin of replication is located at 9 o'clock on the map, between *argG* and *xyl,* with a clockwise direction, thus confirming our previous conclusions;
3. that the origin and direction of vegetative replication are the same for all K12 strains tested (about 12) and for all sexual types (F^+, F^-, F', and Hfr) derived from a single strain;
4. that during amino acid starvation, chromosome replication does not proceed to completion, as is

generally taken for granted, but stops, in a significant number of cells, before the end of the chromosome.

Reference

[1] University of Connecticut, Storrs.

2.9 SEGREGATION OF CHROMOSOME STRANDS IN *ESCHERICHIA COLI*

L. G. Caro

Recent studies (see Sect. 2.8) have demonstrated the existence of multiforked replication in *Escherichia coli* growing in rich medium. This feature of chromosome replication may allow one to distinguish between symmetrical (Cairns' type) and nonsymmetrical (rolling circle) models of DNA replication. It can be expected that a nonsymmetrical replication, coupled with the presence of several growing points on each chromosome, would result in a nonsymmetrical segregation of chromosomal material.

To test this hypothesis we studied, by quantitative autoradiography, the segregation of label in cells of *E. coli* B/r labeled with a pulse of ^{3}H-thymidine and grown in L broth for several generations. The results of this and other similar experiments done at various growth rates indicated that (1) *E. coli* cells growing with a doubling time of 20 min contain an average of two chromosomes with 3 growing points each, while at 30 min doubling time they contain 2.6 growing points per cell (these results agree with the predictions of the Cooper-Helmstetter model for DNA control in *E. coli,* (2) there is little breakage and exchange of chromosome sister strands in cells growing in liquid media, and (3) the segregation of chromosome strands is symmetrical for three generations as predicted by the classical model but not by the rolling circle model.

We conclude that in *E. coli,* DNA is replicated by a symmetrical process and that premature initiation takes place on the two daughter chromosomes at once.

2.10 REPLICATION OF AN F′ FACTOR IN *ESCHERICHIA COLI*

L. G. Caro Claire M. Berg[1]

The replication of the F′ factor ORF-8, carrying the markers *lac-proC-purE,* was studied in partially diploid strains (diploid for the F′ markers) and in haploid strains (with a chromosome deletion of the F′ markers) of *E. coli* W1485. P1 transduction was used to analyze marker frequency, amino acid starvation to obtain partial synchronization of replication, and bromouracil density labeling to determine the extent of replication of a given marker. We found that: (1) the F′ factor is replicated during the late stages of the residual DNA replication occurring during amino acid starvation; (2) it is not replicated during the early stages of the replication occurring when the essential amino acid is restored; (3) in exponential cultures, with an average of three growing points per chromosome, there are 2 to 2.5 copies of the F′ factor per chromosome (this is the frequency expected for a marker located halfway between the origin and the end of chromosome replication). We conclude that the replication of the F′ factor ORF-8 does not coincide with the initiation of chromosome replication but that it occurs approximately during the middle of the chromosome replication cycle.

Reference

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2.11 CONTROL OF CHROMOSOME REPLICATION AND CELL DIVISION BY AN INTEGRATED EPISOME

L. G. Caro Yukinobu Nishimura Claire M. Berg[1]

The strain of *E. coli* CRT46 carries a temperature-sensitive mutation, T46^{-}, located near *ilv* and affecting DNA replication. Hirota has shown that this mutation seems to affect the initiation of chromosome replication. The F^{+} derivative of this strain is also temperature sensitive. We have studied temperature-resistant revertants of CRT46 F^{+}.

In one experiment 79 independent revertants were isolated. Three were low-frequency donors, while 76 were Hfr (in 100 colonies of CRT46 F^{+} grown at 30°, without temperature selection, no Hfr was found). The sites of F integration were not random: 71 were in the lower left quarter of the *E. coli* map, none was located in the right half, all but 4 transferred the chromosome, in mating, in the clockwise direction (the same direction as that of chromosome replication). Seven different sites of integration were identified. Conjugation and P1 transduction showed that in all the Hfr temperature revertants the original T46^{-} mutation was still present. It was concluded that the reversion was due to a new type of suppression, associated with the integration of F in the chromosome, which we call integrative suppression.

It is known that acridine orange inhibits specifically the replication of the F factor. This inhibition does not

apply, however, to the integrated F of an Hfr cell, presumably because the replication of F is then under chromosomal control. In the Hfr temperature-revertant strains, however, chromosome replication is inhibited at 42° by acridine orange. This inhibition disappears at 30° and at 42° does not affect cells carrying a reverse mutation of the T46 site ($T46^+$). We conclude, from these and other experiments, that in the Hfr revertants, replication of the chromosome and cell division are under the control of the episome F. Since this type of reversion requires the integration of the episome into the continuity of the chromosome, we postulate that the chromosome is replicated, in these cells, by the replication machinery of the episome.

Reference

[1] University of Connecticut, Storrs.

2.12 GROWTH OF MONSTER CELLS OF *E. COLI*

D. P. Allison L. G. Caro

We have studied some cytological and physiological effects of the combined mutations *lon*$^-$ (failure of cell division after irradiation) and *mon*$^-$ (formation of shapeless cells) in cells of *E. coli* K-12 P678-A4, a strain isolated and characterized by H. I. Adler.

It was found that environmental conditions such as salt concentration and growth on solid vs liquid media affected the survival rates and formation of monster cells (large, amorphous cells). In liquid medium the formation of monster cells, favored by high salt concentrations, seems to be irreversible and lethal. Electron microscope study of thin sections of these cells grown, after x irradiation, on a rich agar surface showed that as the cells become large there appears an extensive network of intracellular membranes forming vacuoles, cisternae, vesicles, and tubules. These structures bear a striking resemblance to the rough and smooth membranes (endoplasmic reticulum, Golgi complex, vacuoles, etc.) of higher cells. These cells offer an interesting system with which to study the formation and function of intracellular membranes.

2.13 GENETIC RECOMBINATION AT THE MOLECULAR LEVEL

Margaretha Kellenberger-Gujer

General recombination in phage λ, in contrast to the site-specific *Int*-promoted recombination, can be carried out either by the recombination system of the bacterial host cell (*Rec*-system) or by a group of at least three λ genes (*Red*-system). We tested each system separately for DNA exchange during recombination and found that general recombination also involves breakage and reunion, as shown by Meselson and by me, for the *Int*-directed recombination. (*Int* is the phase-directed system needed to integrate λ as a prophage into the chromosome at a specific site.)

We tried to elucidate the mechanism of DNA exchange during general recombination by studying the genetic as well as the physical structure of the recombinants formed by either the *Rec*- or the *Red*-system. We excluded the possibility of single strand exchanges and demonstrated by several means that λ recombination involves a double strand break on each parental chromosome and crosswise, reciprocal rejoining. In other words, λ recombines as a ring, inserting itself in another λ ring to form a dimer ring. The two reciprocal recombinants (physically, but not necessarily genetically reciprocal) are cut out later, during maturation, when the chromosome becomes linear and the sticky ends are formed by the terminase. Making use of λ dv, a little fragment of λ which can multiply autonomously in the bacterial cell without possessing the two ends of the mature λ chromosome, we could demonstrate that recombination inserts the whole λ dv into a phage chromosome. The resulting recombinants carry λ dv as a duplication, as shown by genetical evidences and by the fact that they become heavier, containing more DNA. The reciprocal event, namely, excision of the duplication, also takes place by intramolecular recombination without cutting the genome into two pieces. We propose that the ring-into-ring insertion model is a general system of recombination and applies to systems other than λ and sex factors.

2.14 LACK OF DNA INJECTION BY PHAGES λ AND T4 INTO MINICELLS

Margaretha Kellenberger-Gujer

In an attempt to study the recombination of λ DNA in the absence of extensive multiplication, we found out that λ does not inject its chromosome into purified minicells. This observation holds also for T4D phages. Both λ and T4 adsorb on minicells with the same high efficiency as on normal cells (separated out from the same culture), but after ultrasonic treatment or freezing and thawing followed by slow centrifugation the DNA is found in the supernatant in the case of the minicells and in the pellet in the case of normal cells. Minicells as well as normal cells withstand the short ultrasonic treatment and repeated freezing and thawing treatments.

2.15 THE ROLE OF F PILI IN THE PENETRATION OF THE BACTERIOPHAGE f1

Ann B. Jacobson Dorothea V. Parker

The bacteriophage f1 is a filamentous DNA-containing virus which infects cells of bacteria carrying the sex factor F. Such cells have filamentous appendages known as F pili to which the phages attach. F pili are essential both for the penetration of male-specific phages and for bacterial conjugation, but little is known of their role in these processes. The experiments described here were designed to obtain information on how F pili function during phage infection.

Synchronous penetration was achieved by pre-attaching phage to cells of *E. coli* at 0°C. After appropriate purification the cells were transferred to 37°C. Under these conditions the bulk of the phage DNA entered the cells within 6 min after the cells were transferred to 37°. The entry of f1 DNA into the cells was interrupted at various times during the 6-min interval by the addition of 1% formalin, and the cells were then examined in the electron microscope.

Initial examination of the penetration process was made by electron microscope autoradiography, since it has been suggested by other investigators that the pilus functions only as a tube through which DNA passes on its way into the cell. If true, silver grains should be found over pili while DNA is entering the cell. In the preparations examined to date, silver grains were found only rarely over pili, and almost all grains were associated with visible phage. These observations suggest that f1 DNA does not enter the pilus before entering the cell.

It is also possible that pili are resorbed by the cells during the process of phage penetration. If so, both the number and length of pili with f1 attached might be expected to decrease as phage DNA enters the cell. In one experiment designed to examine this possibility, the average number of phage particles per cell dropped from 1.4 to 0.26 in 6 min at 37°. There was a significant decrease in pili length during this same interval. In addition, the number of cells which have no pili rose from an initial level of 13% at the beginning of the experiment to 35% when most of the phage DNA has entered the cell. Thus it seems likely that pili are resorbed during phage penetration.

2.16 THE ROLE OF DNA IN THE CONTROL OF RESPIRATION BY UV-IRRADIATED BACTERIAL CELLS

P. A. Swenson R. L. Schenley

Ultraviolet radiations cause severe temporary inhibition of respiration and growth in *Escherichia coli* B/r. In mineral salts and glycerol (M63 medium) respiration after a dose of 500 ergs/mm^2 is turned off 60 min after uv and remains so for about 4 hr. Repair of DNA is not necessary for the turning off of respiration after uv radiation, but it is necessary for the resumption of respiration, once inhibition has set in. These points were established by incubating irradiated cells with caffeine, a drug which prevents the excision of pyrimidine dimers from uv-irradiated DNA.

Respiration is not turned off in cells that are treated immediately after uv with chloramphenicol or 5-fluorouracil or are infected immediately after uv with an amber mutant (AmN82) of T4 bacteriophage. If these agents are added after respiration has turned off, respiration does not resume. The results indicate that the uv-damaged DNA must be transcribed to form a messenger RNA, which participates in the synthesis of a protein necessary for the turning off of cell respiration. This interpretation is useful in explaining why respiration does not stop in B/r cells irradiated with massive doses of uv; the extensive damage to DNA may prevent messenger RNA formation.

Survival of cells is about 1% for cells plated immediately after uv. If the cells are incubated at 37°C, survival increases (doubling time about 50 min), beginning at about 150 min after uv. We postulate that all irradiated cells have their respiration turned off, but only those that have it turned on again can survive. When irradiated cells are incubated with 5-fluorouracil for 60 min a dramatic increase in the number of survivors results; the number of survivors is 20 times that for the cells incubated for the same time without 5-fluorouracil. We suggest that the reason for the increased survival is that the 5-fluorouracil causes a coding error in the messenger RNA which codes for a protein responsible for turning off respiration in uv-irradiated cells.

In an effort to find the immediate cause of the inhibition of respiration by uv, we tested the ability of sonified extracts of irradiated cells (90 min after uv) to

carry on cell-free respiration with glycerol and various glycolytic and Krebs cycle intermediates as substrates. Cell-free respiration is almost completely absent for all intermediates employed except for α-glycerolphosphate and succinate. These two substrates are the only ones whose electron transport is not linked through pyridine nucleotides. Addition of DPN and TPN to cell-free preparations stimulates oxygen consumption with substrates such as malate and isocitrate. Oxygen consumption with glycerol, pyruvate, α-ketoglutarate, and oxalacetate was not stimulated by addition of pyridine nucleotides. The data suggest that respiration turnoff is caused by two things: inactivation of glycerol kinase and disappearance of pyridine nucleotides. Addition of α-glycerolphosphate to whole cells 90 min after uv results in respiration but not growth. Analysis of pyridine nucleotide levels in cells at various times after uv showed that these have almost disappeared by 90 min and that the level remains low for the next 3½ hr.

Under conditions where respiration is turned off for various periods after irradiation, there is a good correlation between the rate of respiration and pyridine nucleotide levels in the cells. We have not been able to detect DPNase activity in irradiated cells.

2.17 LOSS OF THE CAPACITY OF UV-IRRADIATED *ESCHERICHIA COLI* B/r TO SUPPORT THE GROWTH OF PHAGE T4

J. M. Boyle P. A. Swenson

The capacity of a phage-infected cell is defined as its ability to give rise to a plaque. When glycerol-grown cells of *E. coli* B/r are uv irradiated and immediately used as hosts for phage T4, inactivation of the capacity of the cells to grow T4 occurs at doses 100 times those required for inactivation of the cells. When the same cells are inactivated to 1% survival with 500 ergs/mm^2 of uv and subsequently incubated in glycerol medium at 37°, loss of capacity commences after about 30 min, and by about 90 to 120 min only 3% of the cells retain capacity. At later times the cell population slowly recovers capacity. Loss of capacity occurs in those cells destined not to survive uv irradiation; the slow recovery of capacity is due to multiplication of surviving cells in the irradiated population.

In a comparison using nonirradiated cells, cells irradiated immediately prior to testing, and irradiated cells incubated for 90 min prior to testing, we demonstrated that T4 both adsorbs and injects its DNA equally well in each case. In cells which lose capacity, T4 DNA is neither degraded nor replicated, and there is apparently very little transcription and translation of T4 DNA since T4-induced degradation of host DNA does not occur.

We conclude that loss of capacity results from loss of a cytoplasmic function of the host cell which is required for transcription and translation of an undamaged viral genome. From concurrent studies it seems likely that this function is the provision of an energy source and its loss is related to the lowering of pyridine nucleotide levels in irradiated cells. This conclusion is strengthened by the fact that 5-fluorouracil and rifampin, which prevent the turnoff of respiration, markedly limit the loss of capacity.

2.18 COMPETITION FOR EXCISION ENZYMES BY DIMERS IN VIRAL AND CELLULAR DNA'S

J. M. Boyle R. B. Setlow

The excision and repair of uv-irradiated pyrimidine dimers in the DNA of a bacterial virus, phage λ, is mediated by host-cell enzymes.[1] In cells containing either irradiated λ DNA *or* irradiated bacterial DNA, bacterial dimers are excised at a rate 30 times faster than λ dimers. Contrastingly in cells containing dimers in both λ DNA and bacterial DNA, dimers in λ DNA limit the excision of bacterial dimers better than in the converse situation.

To accommodate both of these observations we propose that there may be an unequal distribution of excision enzymes in a cell. The endonuclease, which initiates excision, may occur more or less randomly within the cell, but the exonuclease activity, which results in dimer excision, may be localized near bacterial DNA. This hypothesis leads to a testable interpretation of ultraviolet reactivation (uvr) – the increase in survival of irradiated phage particles when assayed on cells that have been lightly irradiated, compared with the survival observed when assays are made on nonirradiated cells. Harm[2] explained uvr in terms of the competitive removal of degrading nucleases that impair excision of dimers in phage DNA by the introduction of lesions in bacterial DNA. According to our hypothesis, dimers in bacterial DNA would compete for endonuclease molecules, thus reducing the number of single-strand breaks (sites for nonspecific exonuclease activity) but not markedly reducing the excision of λ dimers, since the rate-limiting step here is that of the excision-specific exonuclease. We observe that uvr is associated with a small decrease, rather than an increase, in the number of λ dimers excised.

References

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[2] W. Harm, p. 107 in *Repair from Genetic Radiation*, ed. by P. H. Sobels, Pergamon Press, London, 1963.

2.19 EXCISION-REPAIR PROPERTIES OF A MUTANT OF *ESCHERICHIA COLI* DEFICIENT IN DNA POLYMERASE

J. M. Boyle M. C. Paterson[1] R. B. Setlow

Although considerable evidence has been presented concerning the validity of the proposed excision repair system for uv-induced lesions in DNA and although enzymes (uv endonuclease and polynucleotide ligase) which may catalyze the first and last steps in the reaction have been isolated, virtually nothing is known of the identity of the enzyme which actually excises uv lesions. Recent in vitro studies with purified DNA polymerase molecules from *E. coli* suggest this enzyme as a possible candidate.[2] We have compared certain excision repair properties of a uv-sensitive mutant (P3478) of *E. coli*, extracts of which lack DNA polymerase activity,[3] with those of its parent, W3110 thy^-.

The mutant grows normally and is five times more sensitive to both uv and x irradiation than W3110. The increased sensitivity to radiation is not due to an inability to excise radiation-induced lesions, since excision of pyrimidine dimers is only slightly impaired in P3478. The increased sensitivity appears related to an enhanced nuclease activity which causes 85% of the DNA of P3478 to become acid-soluble following a dose of 250 ergs/mm^2, a dose which renders only 20% of the DNA of W3110 acid soluble. At a constant survival level the DNA of both strains is solubilized to approximately the same extent. Alkaline sucrose gradient analysis of the DNA of irradiated cells indicates (1) that acid solubilization of the DNA of P3478 is not produced by a rampant endonuclease and (2) that some repair of irradiated P3478 DNA does occur. Our results are consistent with the idea that P3478 possesses an enhanced exonuclease which acts at a single-strand DNA terminus produced either by a uv endonuclease or by x rays. The mutation in P3478 appears to have resulted in properties which are analogous to those of a preparation of purified DNA polymerase which was chemically modified by acylation. In vitro the modified enzyme has 0.2% of the polymerase activity and 920% of the exonuclease activity of the normal enzyme.

References

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

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2.20 REPAIR OF PHOTOPRODUCTS BY VEGETATIVE CELLS

J. E. Donnellan, Jr. R. S. Stafford

When DNA in solution or in vegetative cells is irradiated in the frozen state, a different spectrum of photoproducts is produced. In particular, a photoproduct identical in chromatographic properties to the photoproduct found in bacterial spores becomes the dominant thymine product, accounting for more than half the thymine-containing photoproducts, the additional products being cyclobutyl pyrimidine dimers. We have examined the repair of cyclobutyl pyrimidine dimers and spore-type photoproducts following irradiation of cells at −196°C.

The types of cells studied, *Bacillus megaterium*, *Escherichia coli*, and *Micrococcus radiodurans*, exhibit efficient excision mechanisms when irradiated in solution. The first two retain the excised cyclobutane-type dimers within the cell (acid soluble), while these dimers are excreted into the medium by the last organism. The organisms, however, conserve the dimers in that all dimers can be accounted for after the time allotted for repair. This differs from the repair of spore photoproduct in germinating bacterial spores in that the spore product disappears, presumably by reversion to thymine.

Following irradiation at −196°C and repair at 37° in vegetative cells all photoproducts could be accounted for in *E. coli* and *M. radiodurans*. In *M. radiodurans* the excision and excretion of spore-type product appeared to be identical to that of the cyclobutane product. In *E. coli*, however, the spore-type product was excreted into the medium, while the cyclobutane product was retained in the acid-soluble fraction of the cell. On the other hand, in vegetative cells of the spore former, *B. megaterium*, the spore-type photoproduct disappeared relative to cyclobutane dimers, although some of the spore-type products were also excised and appeared in the medium.

The excision of spore-type photoproduct by vegetative cells must be different in part from the excision of cyclobutyl pyrimidine dimers to allow the products to be excreted into the medium. The vegetative cells of a

spore-forming bacterium seem to retain the property of the germinating spore in that some of the spore-type products disappear.

2.21 THE SPORE PHOTOPRODUCT

J. E. Donnellan, Jr. R. S. Stafford

The irradiation of bacterial spores in aqueous suspension with uv light produces thymine photoproducts which are chromatographically different from the cyclobutane thymine dimers produced in vegetative cells and in DNA in solution. This observation strongly suggests that the state of DNA in spores is different from that in vegetative cells. A knowledge of the nature of the spore photoproducts would help in elucidating the state of this DNA. Spore photoproducts are also produced, along with cyclobutane dimers, upon irradiation of dry films of DNA or thymine nucleosides or nucleotides. Approximately 8 mg of spore products have been isolated from 600 mg of dry irradiated TMP with a ^{14}C label by paper and column chromatography following acid hydrolysis. The product has a specific radioactivity equal to that of the starting material and, by cochromatography in several solvents, consists solely of material indistinguishable from the major spore product. Uv, infrared, and NMR spectra have been obtained. The uv spectrum appears identical to that of thymine, although the absorbance per unit mass is reduced to one-half. The infrared and NMR spectra, while exhibiting some similar peaks, also exhibit differences. In particular, the methyl resonance in the NMR spectra is shifted to a δ of about 1.0 ppm.

A proposed dimer containing an acetidine ring and one unsaturated 5-6 bond, while compatible with the uv spectrum, appears to be ruled out by the NMR spectrum. Additional analysis of this product must be made before a structure can be assigned.

2.22 MEASUREMENT OF DNA REPAIR IN SUBCELLULAR ORGANELLES

J. S. Cook T. E. Worthy

We have been developing sensitive chromatographic methods for measuring ultraviolet (uv) lesions in DNA. Our principal concern has been with techniques for measuring small quantities of pyrimidine dimers, caused by biologically significant doses of uv, in DNA's which cannot be labeled with specific precursors such as ^{3}H-thymidine; our goal is to measure repair in such DNA-containing organelles as mitochondria from yeast and *Neurospora*. The basis of the assay is the competition by uv-irradiated DNA (unlabeled) in the monomerization of ^{3}H-thymine-containing dimers in labeled *E. coli* DNA by photoreactivating enzyme and light. Although the assay has been known for some time, we have substantially enhanced its sensitivity and convenience by using nanogram quantities of DNA of very high specific activity in which a large fraction of the thymine has been converted to dimer either with uv or in a photosensitized reaction with acetophenone. Dimers in such DNA are readily quantitated in one-dimensional chromatography. Using conditions in which the monomerization reaction is first-order, the rate of photoreactivation of labeled dimers is dependent on the quantity of, uv dose to, or base composition of the competing DNA; from any two of these parameters the third may be estimated from the reaction rate under standardized conditions. We are using the same conditions to quantitate repair in DNA isolated from organelles.

2.23 ULTRAVIOLET ACTION SPECTRUM FOR THE PRELYTIC MEMBRANE DEFECT IN HUMAN ERYTHROCYTES

J. S. Cook

The nonnucleated human red blood cell (RBC) has been extensively used in membrane studies because of its relatively simple cytological structure as well as its availability. From previous studies on the action of ultraviolet (uv) radiation on RBC membranes, two widely accepted conclusions have been drawn: (1) cell membranes are relatively insensitive to uv and (2) the action spectrum for hemolysis resembles an "end-absorption" curve, which is consistent with the hypothesis that lipids are the responsible light absorbers. However, most of this older work was carried out under unsatisfactory optical conditions, and all of it was done before either the kinetics or the mechanism of hemolysis was known. Having recently demonstrated dose-rate independence and dose-squared kinetics over a wide range of uv doses and having shown that hemolysis is a much delayed but nevertheless direct consequence of a few electrolyte "leaks" in the cell membrane, I have redetermined the action spectrum. It has a peak at 280 nm, has a minimum near 265 nm, and rises steeply below 248 nm. The shape is characteristic of absorption by aromatic residues in proteins, although absorption by cystine has not been ruled out. Lysis in 24 hr is caused by 5000 ergs/nm^2 at 280 nm, from which it may be calculated that substantial electrolyte leaks will be caused by as little as 500 ergs/nm^2 at this wavelength.

2.24 THE USE OF ALKALINE SUCROSE GRADIENTS TO SEDIMENT THE DNA FROM MAMMALIAN CELLS

R. B. Setlow

The detection of small numbers of single-strand breaks in the DNA of cells as a result of x irradiation or of excision of photoproducts after uv irradiation is only possible if DNA can be isolated and analyzed with little shear degradation. McGrath and Williams[1] developed a technique, useful for bacterial cells, in which radioactive cells are lysed and the DNA denatured by slowly adding cells to an alkaline layer on top of an alkaline sucrose gradient in a centrifuge tube. Subsequent centrifugation separates the molecules (detected by their radioactivity) in terms of their sedimentation velocities. The technique does not work well for mammalian cells because aggregation among DNA, protein, and membrane components results in the DNA sedimenting in hypersharp bands of variable sedimentation rate even at low cell numbers (2000 cells per 3.7 ml of gradient). At high cell numbers DNA and protein form a gel which sediments rapidly to the bottom.

A simple modification of the McGrath and Williams technique – the use of alkaline gradients in which the salt concentration is increased to 2 *M* NaCl – results in the dissociation of DNA from other cellular components. This yields reproducible sedimentation of DNA molecules (even at cell numbers up to 10,000 per gradient) whose sedimentation constants correspond to molecular weights of $1-3 \times 10^8$.

Reference

[1] R. A. McGrath and R. W. Williams, *Nature* **212**, 534 (1966).

2.25 THE CRYSTAL STRUCTURE OF 1-METHYL-4-THIOURACILYL-*p*-MERCURIBENZOIC ACID

S. W. Hawkinson B. C. Pal J. R. Einstein

4-Thiouridine, a minor nucleoside present in *E. coli* tRNA, offers a unique site for the attachment of –SH-specific heavy-atom reagents. With this object in mind the product of the reaction between the methyl analog of 4-thiouridine and *p*-chloromercuribenzoic acid has been crystallized and examined by three-dimensional x-ray analysis.

1-Methyl-4-thiouracilyl-*p*-mercuribenzoic acid (I) was obtained in 60% yield by treating 1-methyl-4-thiouracil with *p*-mercuribenzoic acid; mp 238°d. Spectral properties at pH 7, phosphate buffer: λ_{max} 310 nm and ϵ_{max} 17,960. Analysis calculated for $C_{12}H_{10}O_3N_2HgS$: C, 31.13; H, 2.18; N, 6.05. Found: C, 31.68; H, 2.18; N, 6.25. The compound I crystallizes from 5% ammoniacal solution as thin, rectangular plates. The crystals are orthorhombic, space group *Pbca*, with unit cell dimensions $a = 16.015(2)$, $b = 13.618(1)$, $c = 11.797(1)$ A, and $Z = 8$. The structure was solved by Patterson and Fourier methods, using 1320 intensities measured with an Oak Ridge computer-controlled x-ray diffractometer. Full-matrix least-squares refinement has yielded an *R* value of 0.085 at the present stage in the analysis.

The crystal consists of dimers situated about centers of symmetry and held together by OH · · O hydrogen bonds involving a carboxyl group and a carbonyl oxygen of the pyrimidine ring. The benzene and pyrimidine rings are nearly coplanar, and the C–S–Hg angle of 103°, combined with the linear S–Hg–C bonding, gives the molecule an L-shaped appearance. The distances C–S = 1.75 A, S–Hg = 2.34 A, and Hg–C = 2.03 A are all normal covalent bonds, while the OH · · O hydrogen bond distance of 2.56 A is typical of carboxylic acids.

2.26 CRYSTALLIZATION OF tRNA

C. T. Butler[1] J. R. Einstein

Attempts are being made to crystallize tRNA in a form suitable for x-ray diffraction studies. The technique being employed is based on one developed at the University of Wisconsin in which equilibration of the sample solution with concentrated ammonium sulfate solutions *via* the vapor phase brings about precipitation of the tRNA. The first experiments have been done with samples of $tRNA^{fMet1}$ and $tRNA^{fMet2}$ from *E. coli* B, made available by G. D. Novelli and prepared as concentrated solutions of the magnesium salts by M. P. Stulberg. Equilibration of 5- to 10-μl droplets, containing tRNA, ammonium sulfate, Tris buffer (pH 7.6), and in some cases other divalent cations besides magnesium, was carried out in specially built chambers at three temperatures: 4, 22, and 37°C. Crystals have been obtained at 4 and 22° in the form of very thin plates or very short rods, neither of which are suitable for x-ray work. Attempts are continuing with samples of a number of tRNA's from *E. coli* K12, M0, and M07.

Reference

[1] Solid State Division.

2.27 PRELIMINARY CRYSTALLOGRAPHIC DATA FOR THE VARIABLE PORTION OF A HUMAN BENCE-JONES PROTEIN[1]

J. R. Einstein C. H. Wei

Several preparations containing crystals of the variable portion (V_L) of a human Bence-Jones protein, obtained from a single multiple myeloma patient, were obtained from Dr. Alan Solomon of the University of Tennessee Memorial Research Center, Knoxville. Although crystals from one preparation were twinned, a crystal from another preparation was not and gave diffraction patterns extending beyond $3\frac{1}{2}$-A spacings. (The maximum resolution of the patterns has not yet been determined.) The crystal is monoclinic, space group $P2$, with cell dimensions a = 65.6 A, b = 37.9 A, c = 43.6 A, and β = 90.0°. Assuming the water content of the crystal and the partial specific volume of the protein to be typical, the asymmetric unit would contain approximately 21,500 daltons. Given that the molecular weight of the V_L protein is roughly 11,000, the asymmetric unit probably contains two protein molecules. A structure determination of this protein depends on obtaining suitable heavy-atom derivatives.

Reference

[1] Research supported jointly by the National Cancer Institute and the U.S. Atomic Energy Commission under contract with Union Carbide Corporation.

2.28 THE CRYSTAL AND MOLECULAR STRUCTURES OF *N*-CHLOROACETYL- AND *N*-BROMOACETYL-L-PHENYLALANYL-L-PHENYLALANYL ETHYL ESTER[1]

C. H. Wei D. G. Doherty J. R. Einstein

The crystal structures of the title compounds ($C_{22}H_{25}N_2O_4X$, X = Cl or Br) have been determined by three-dimensional x-ray analysis. The compounds were synthesized as part of a general study of the kinetics of pepsin hydrolysis of synthetic substrates. Crystallographic studies were undertaken because knowledge of the three-dimensional conformation of this substrate is useful in understanding the complementary surface of the active center of pepsin.

The crystals, obtained from ethanol solutions, are monoclinic with cell parameters, for the chloro derivative, a = 12.844(3) A, b = 17.677(4) A, c = 4.931(1) A, and β = 103.30(2)° and, for the bromo derivative, a = 12.951(9) A, b = 17.617(26) A, c = 4.951(3) A, and β = 103.23(7)°. The space group in both cases is $P2_1$, with two molecules per unit cell. The data were collected on an Oak Ridge computer-controlled diffractometer with Cu $K\alpha$ radiation to a minimum spacing of 1.1 A. The isomorphous structures were solved by Patterson and Fourier methods and have been refined by full-matrix least squares to $R(F)$ values of 0.105 for the chloro derivative and 0.090 for the bromo derivative. The refinements were based on the 29 nonhydrogen atoms, of which the ethoxy group and the two phenyl rings were treated as rigid groups, with standard bond lengths and angles. All atoms received individual isotropic thermal parameters, except for the halogen atoms, the thermal motions of which were treated anisotropically. Since (1) only a relatively small number of data were obtainable, about 500 reflections (each derivative) for which $F_0^2 \geqslant 3\sigma(F_0^2)$, and (2) the ethyl carbon positions were evidently subject to disorder, which could not successfully be incorporated into the model, the results are relatively imprecise: ±0.04 A for bond lengths and 3° for bond angles. However, the general molecular conformation and the torsion angles which define that conformation have been determined with sufficient precision for informative comparisons with other peptide structures. Since the molecular structure does not differ significantly between the two derivatives, numerical results for the chloro derivative alone are given below.

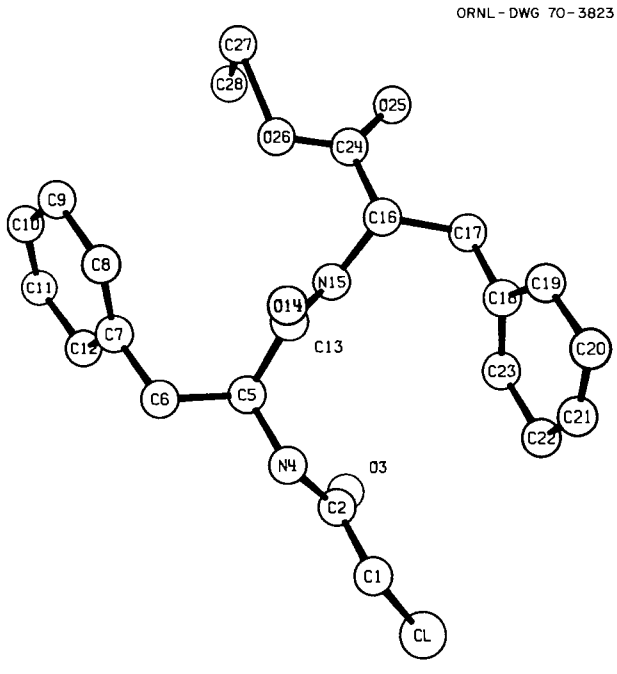

Fig. 2.28.1. Molecular Configuration of *N*-Chloroacetyl-L-phenylalanyl-L-phenylalanyl Ethyl Ester.

The molecule (Fig. 2.28.1) contains two peptide units[2] in an arrangement closely related to the parallel-chain pleated sheet (PCPS) structure. NH · · · O hydrogen bonds connect neighboring peptide units to form a singly pleated sheet extending in the *c* direction. The two phenyl rings, located on opposite sides of the sheet, are nearly parallel and are arranged in infinite stacks in the *c* direction with phenyl rings of adjacent molecules. The molecule is roughly in the form of an "H," with the two phenyl rings at the ends of diagonally opposite "legs," the chloroacetyl and ethyl groups at the ends of the other two legs, and the amide bond linking the two residues as the central "crossbar."

The peptide units are in the *trans* configuration and are planar to within experimental error. The angle of 106.3° between the two peptide planes is in accord with the value of 106.5° for the PCPS structure.[3] The main-chain torsion angles fall within the range of values previously observed for peptides having the PCPS structure.

The three ideal positions for a C^{γ} atom have been called I, II, and III for the possible torsion angles χ^1 about the C^{α}–C^{β} bond.[4] The values of χ^1 determined for residues 1 and 2 are within 5° of position II and within 11° of position III respectively. This is the first known case of position III for a small peptide, and the conformation is important in making this molecule as compact as it is. The values of the torsion angle χ^2, defining the angle of the phenyl plane to the plane C^{α}–C^{β}–C^{γ}, are close to 90°, in agreement with results for other phenylalanyl peptides.

References

[1] Research supported jointly by the National Cancer Institute and the U.S. Atomic Energy Commission under contract with Union Carbide Corporation.

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2.29 ELIMINATION OF SPOT DOUBLING IN PRECESSION PHOTOGRAPHY WITHOUT LAYER-LINE SCREENS

J. R. Einstein

An efficient method of collecting x-ray diffraction data for macromolecular crystals is the recording of contiguous oscillation or precession photographs, taken without layer-line screens, followed by automatic digital processing of the films. One difficulty associated with this technique is the general doubling of spots from all reciprocal-lattice layers except that for which the film is set. Spot doubling is detrimental because (1) integration is required over a larger area, which is generally of more complicated outline; (2) the effective intensity-to-background ratio is decreased; and (3) overlapping of neighboring reflections is more likely. Spot doubling may be eliminated through the use of a mechanical device based on a principle[1] utilized in the photography of single reciprocal-lattice layers. A rotating screen with an open half-circle aperture may be placed in front of the film and driven near its circumference, with suitable gears and a flexible shaft coupling, in a 1:1 ratio with the main drive shaft of the precession motion. Besides eliminating spot doubling, the screen also reduces by half the registration of background radiation. This work was done in collaboration with members of Bioengineering and the Biology Division machine shop.

Reference

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2.30 X-RAY STRUCTURAL ANALYSES OF SOME TRANSITION METAL COMPLEXES[1]

C. H. Wei

Structure of $[Ni\{Ni(NH_2CH_2CH_2S)_2\}_2]Cl_2$. – The diverse stereochemical behavior of the biologically important mercaptan group as a donor in the formation of a large variety of transition-metal sulfur complexes makes this ligand of particular interest. A complete structural determination (including all 12 hydrogen atoms) of $[Ni\{Ni(NH_2CH_2CH_2S)_2\}_2]Cl_2$ (kindly furnished by Drs. D. H. Busch and D. C. Jicha of the Ohio State University) has been carried out. The framework of the diamagnetic cation may be seen as arising from the chelation of two identical $Ni(NH_2CH_2CH_2S)_2$ entities of *cis* configuration to a third Ni(II) atom by four Ni–S bonds, such that this central Ni(II) is surrounded by four planar bridging sulfur atoms. Because of the steric requirements of the chelating $(NH_2CH_2CH_2S)$ ligands, the central planar NiS_4 unit joins each of the two essentially planar NiN_2S_2 units along the S–S line with a dihedral angle of 109°. The resulting $Ni\{NiN_2S_2\}_2$ entity ideally possesses a chair conformation of C_{2h}-2/*m* symmetry. All hydrogen atoms were found to be tetrahedrally attached to carbon and nitrogen atoms.

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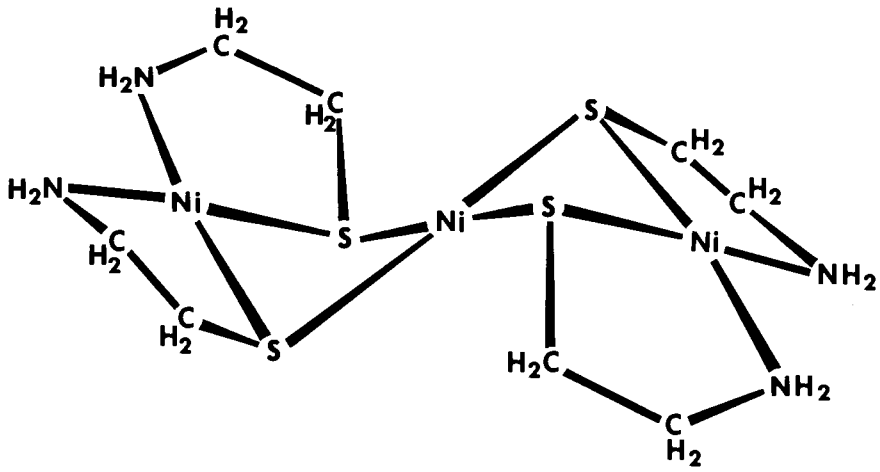

Fig. 2.30.1. $[Ni\{Ni(NH_2CH_2CH_2S)_2\}_2]^{2+}$ Cations.

The ionic compound forms triclinic crystals with one formula unit in a centrosymmetric primitive unit cell with parameters a = 5.29(1) A, b = 8.55(2) A, c = 11.19(2) A, $\alpha = 110°28'(10')$, $\beta = 96°32'(10')$, and $\gamma = 91°50'(10')$. The structure was solved by three-dimensional Patterson and Fourier methods and refined by full-matrix least-squares methods to $R_1(F) = 4.9\%$ and $R_2(F) = 5.4\%$, respectively, based on 1213 independent intensity maxima obtained photographically. The crystal structure was shown to be partially stabilized by a hydrogen-bonding network involving the amino groups of the $[Ni\{Ni(NH_2CH_2CH_2S)_2\}_2]^{2+}$ cations (Fig. 2.30.1) and the chloride ions.

Structures of bis(*N*-*t*-butylpyrrole-2-aldimino)-metal(II), $(C_9H_{13}N_2)_2M(II)$. – It has been known that tetradentate metal complexes can be derived from Schiff bases. Due to the great synthetic flexibility of Schiff base formations, numerous complexes have been prepared with a variety of combinations of basic ligands and permutable metals. It is of interest to study the configuration of the metal environment as it depends on the counterbalancing of (1) steric stresses imposed by the choice of ligand and (2) the inherent configurational tendency of the various metals. The title complexes, generously made available by Dr. R. H. Holm of MIT, are of some biochemical interest, inasmuch as the pyrrole-2-aldimine group is an isosteric fragment of pyrromethenes and porphyrines.

Three-dimensional x-ray investigations of bis(*N*-*t*-butylpyrrole-2-aldimino)metal(II) complexes (with metal = Co and Ni), metal complexes of a Schiff base, have been completed. These two complexes appeared by their diffraction patterns to be isomorphous, but the detailed structural analyses revealed them to be not truly isostructural. The molecular configuration of C_2-2 symmetry for each of these two complexes consists of two bidentate ligands coordinated to the central metal atom via four M–N bonds.

The cobalt complex crystallizes with four formula species in an orthorhombic unit cell of symmetry *Pbcn* and dimensions a = 17.20(2) A, b = 7.18(1) A, and c = 15.23(2) A. The configuration around the cobalt atom is tetrahedral, with a dihedral angle of 90° between the planes of the pyrrole-2-aldimine ligands. The structure of this complex (including all 13 hydrogen atoms) has been refined to give $R_1(F) = 7.1\%$ and $R_2(F) = 6.3\%$, based on 656 independent observed photographic data. Crystals of the nickel complex have a unit cell of dimensions a = 16.777(13) A, b = 7.507(6) A, and c = 15.378(10) A. The configuration around the nickel atom is nearly tetrahedral, with a dihedral angle of 89° between the ligands. A salient feature of this structure is that the *t*-butyl group appears to possess two alternative orientations, one of which is similar to that found for the cobalt complex. This partially disordered structure was refined by means of a rigid-group least-squares technique, which yielded $R_1(F)$ and $R_2(F)$ values of 7.1 and 8.0%, respectively, based on 648 independent counter data.

Details of structural analyses for the cobalt and nickel complexes provide an inclusive comparison of these two structures with those previously determined for two analogous copper(II) complexes, for which the equivalent dihedral angles are 61 and 59°.

Reference

[1] Research supported jointly by the National Cancer Institute and the U.S. Atomic Energy Commission under contract with Union Carbide Corporation.

2.31 HALOACETOL PHOSPHATES: POTENTIAL ACTIVE-SITE REAGENTS FOR ALDOLASE, TRIOSE PHOSPHATE ISOMERASE, AND GLYCEROPHOSPHATE DEHYDROGENASE

F. C. Hartman

Preparation and Properties. – Chloro-, bromo-, and iodoacetol phosphate, reactive derivatives of dihydroxyacetone phosphate, were synthesized as potential active-site reagents for aldolase, triose phosphate isomerase, and glycerophosphate dehydrogenase. The dimethyl ketals of the reagents were obtained as crystalline bis-cyclohexylammonium salts by a series of reactions starting with 3-halo-1,2-propanediols. At pH 10.2, haloacetol phosphates are rapidly converted to dihydroxyacetone phosphate, but at pH 6 to 8 are stable for hours and even days in the case of the chloro derivative. Sulfhydryl is the only functional group of free amino acids that reacts with haloacetol phosphates. The SH group of glutathione is covalently modified by chloro- and bromoacetol phosphate to form a presumed S-alkyl derivative. Iodoacetol phosphate oxidizes sulfhydryls predominantly to disulfides. These studies suggest that haloacetol phosphates will react preferentially with SH groups of proteins.

Inactivation of Aldolase. – In an effort to find active-site-specific reagents for fructose diphosphate aldolases, I investigated the reaction of haloacetol phosphates with rabbit muscle aldolase. Under mild conditions, iodoacetol phosphate (IAP) rapidly inactivates aldolase, but bromo- and chloroacetol phosphate have no effect. Twenty-one of the twenty-nine free sulfhydryl groups in aldolase disappear during its inactivation with IAP. Incubation of the inactivated enzyme with cysteine restores 65% of the activity and all but two to three of the sulfhydryl groups. Sulfite or cyanide restores about 50% of the activity and 50% of the sulfhydryl groups restored by cysteine, demonstrating that the major modification is an oxidation of sulfhydryl groups to disulfides. The irreversible losses of enzymic activity and sulfhydryl groups correlate with the covalent incorporation of two moles of reagent per mole of aldolase. The lack of incorporation in the presence of substrate implies that the labeled groups are essential.

In the presence of substrate, 12 sulfhydryl groups of aldolase are oxidized with no loss of activity; but upon removal of the substrate from the solution containing the active, modified aldolase, 65% of the activity is lost. Experiments in which arsenite was used as a reducing agent suggest that in the presence of substrate, IAP oxidizes two nonessential sulfhydryl groups to sulfenic acids. Inactivation, which occurs upon removal of substrate, is probably the result of essential sulfhydryl groups, which had been protected by substrate, reacting with the sulfenic acids to form disulfides.

Active-Site Modification of Triose Phosphate Isomerase. – I have reported iodoacetol phosphate (IAP) to be an active-site-specific reagent for rabbit muscle triose phosphate isomerase (TPI).[1] The corresponding chloro (CAP) and bromo (BAP) reagents also react specifically with the active site of TPI. At pH 6.5 and 2°, bimolecular rate constants for the inactivation of TPI by IAP, BAP, and CAP are 260, 2600, and 2300 M^{-1} sec^{-1} respectively. Autoradiograms of peptide maps from TPI inactivated with ^{32}P-labeled haloacetol phosphates demonstrate that all three reagents react with a single, identical site. Further confirmation of active-site modification is that CAP inactivates TPI from all species that have been tested (spinach, *E. coli,* yeast, mouse liver, and human whole blood). From a tryptic digest of CAP-modified rabbit-muscle TPI, a peptide containing the incorporated reagent has been isolated. The amino acid composition of this active-site peptide is trp, lys, thr, glu, pro, gly_2, ala_2, val_2, ile, leu, tyr. Glutamic acid has been identified as the labeled, essential residue.

Reference

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2.32 FLUORESCENCE POLARIZATION SPECTRA OF POLYPEPTIDES AND POLYNUCLEOTIDES: A SINGLET ENERGY TRANSFER STUDY[1]

J. W. Longworth Maria Del Carmen Battista

Electronic energy transfer between molecules can be detected from measurements of the fluorescence polarization. When the molecules are side chains of polymers with repeating units that form helical conformations

such as polypeptides and polynucleotides, no two neighbors will have identical coordinates. The absorption of polarized light by one side-chain moiety and emission by another must cause a depolarization, as opposed to absorption and emission by the same molecule. The principle applies whether the molecules are identical or dissimilar. A considerable depolarization has been observed for poly-L-tyrosine, for all wavelengths of absorption except at the long-wavelength limit, where no depolarization is found. We have extended these observations to other polypeptides, where tryptophan is the emitting molecule: $(\text{L-trp})_n$, $(\text{L-trp}_1,\text{D-trp}_1)$, $(\text{L-trp}_1,\text{L-tyr}_1)_n$, and $(\text{L-trp}_1,\text{L-tyr}_4)_n$. We have observed essentially similar results to the poly-L-tyrosine system. There is a considerable depolarization, with a progressive polarization increase at the long-wavelength limit, reaching the value of the monomer. Suitable dimers have also been studied, and these show an intermediate behavior between monomer and polymer. The same pattern of depolarization, except at the long-wavelength limit, is also found for ionized tyrosine emission from poly-L-tyrosine, and the two tyr-trp heteropolymers at pH 12.

Encouraged by these results, we attempted to measure the fluorescence polarization spectra of DNA and related synthetic polynucleotides. Because these molecules have low quantum efficiencies at 220°K, the lowest temperature attainable in our apparatus, we have used denaturing conditions which enhance the quantum yields over those found for the stacked conformations. Poly G and DNA at pH 2, which emit from their protonated G residues, have similar polarization spectra, and this is depolarized compared with that of GMP. A similar behavior is observed for poly A and DNA at pH 12 compared with A. The random coil conformation of DNA and $d(A\text{-}T)_n$ has also been studied at pH 7; these too have a polarization spectrum which is depolarized from the monomer values. Several dinucleotides have been investigated, and the depolarization is greatest in stacked conformations and in dipurine dinucleotides. The maximum polarization values reached in the polymers, though depolarized from monomer values, are comparable with the values of stacked dinucleotides. Hence, we conclude that there is singlet energy transfer in polynucleotides, though only over a small number of bases.

Reference

[1] Research supported jointly by the National Cancer Institute and the U.S. Atomic Energy Commission under contract with Union Carbide Corporation.

2.33 FLUORESCENCE POLARIZATION SPECTRUM OF RIBONUCLEASE T_1 [1]

J. W. Longworth

The fluorescence polarization spectrum of indole in a hydrocarbon is anomalous and can be interpreted by the presence of emission from two electronic levels within the single fluorescence band envelope. It is of interest to investigate the problem with the indole in other environments. The protein ribonuclease T_1 of *Aspergillus oryzae* possesses a single tryptophan, and the fluorescence of the protein is only from this residue. It is characterized by a distinctly fine-structured emission. The spectrum is clearly that of an indole in a hydrocarbonaceous environment, and the fine structure is more distinct than that observed from indole itself. The presence of nine tyrosine residues in the protein produces complications, but excitation studies have shown that only one-third of the energy absorbed by the tyrosines is transferred to the tryptophan residue. When the polarization spectrum of trptyr or tyrtrp (which are similar) is compared with that of $NAcTrpNH_2$, only a small depolarization (~10%) is found, and the trp fine structure is still clearly defined. The effect of a larger tyr/trp ratio is expected to merely increase this depolarization and not drastically alter the spectral features. The polarization spectrum of RNase T_1 is unlike that of trp; it is considerably depolarized, has no fine structure, and becomes progressively more polarized at wavelengths longer than 290 nm. A similar spectrum is found for the endonuclease of *Staphylococcus aureus* (1 trp, 7 tyr), a protein with a similar fluorescence at 220°K, the temperature of these measurements.

The polarization spectra of these proteins are remarkably like the polarization spectrum of the random polypeptide $(tyr_4, trp_1)_n$; here the emission is from the tryptophanyl residues, though the emitting residue has a different orientation from the one that absorbed the exciting photon because of transfer along a helical structure (again there is the complication of partial tyrosyl to tryptophan transfers). In RNase T_1 there cannot be emission from different residues with different orientation; the different orientations have to be provided within a single molecule. This then suggests that a dual-level emission occurs from the tryptophan residue in RNase T_1.

Reference

[1] Research supported jointly by the National Cancer Institute and the U.S. Atomic Energy Commission under contract with Union Carbide Corporation.

2.34 INTRAMOLECULAR TRIPLET-TRIPLET ENERGY TRANSFER: A DELAYED-FLUORESCENCE STUDY[1]

J. W. Longworth Maria Del Carmen Battista

When two triplet states collide with each other, for example by a migration along a polymer chain, they annihilate one another and in so doing form the parent singlet states, of which one is an excited state. The result is the emission of the fluorescence with a long lifetime – a delayed fluorescence – whose intensity depends on the square of the exciting light intensity. We have found such a delayed fluorescence from poly-L-tyrosine at 77°K, and the intensity was dependent on the square of the phosphorescence intensity, which in its turn is dependent directly on the light intensity. The contribution of the delayed fluorescence to the total fluorescence did not reach more than 1% at the maximum light intensities available in our instrument. The lifetime of the delayed fluorescence was found to be exponential in character, with a decay constant of 0.3 sec. We have observed that there is no delayed fluorescence from the oligopeptide hexatyrosine – known to have a helical conformation at low temperatures. Thus triplet migration must be over more than 6 amino acid residues. Since the smallest polymer investigated was 100 residues long and showed delayed fluorescence, the extent of migration must be less than or at least equal to this valve. A similar delayed fluorescence has been found for polyadenylic acid in a single-strand stacked conformation at pH 7.

Hence we conclude that there is triplet-triplet energy transfer in both these polymers.

Reference

[1] Research supported jointly by the National Cancer Institute and the U.S. Atomic Energy Commission under contract with Union Carbide Corporation.

2.35 IS THE FLUORESCENCE OF INDOLE DISSOLVED IN HYDROCARBON SOLVENTS FROM TWO ELECTRONIC LEVELS?[1]

J. W. Longworth J. A. Knopp
Maria Del Carmen Battista

This question has been raised by a variety of experiments from different workers but has no satisfactory answer. Suffice it to say, it would represent a unique photophysical phenomenon, with indole providing the only reported example for the behavior. Any study of indole fluorescence is complicated by the ease of exciplex formation by indole and by the ability of indole to form ground-state hydrogen-bonding complexes. For example, hydrocarbons have to be carefully dried to remove water before the intrinsic fluorescence of the indole is observed. We have chosen two situations for observing the fluorescence of indole: (1) in a hydrocarbon (mineral oil), where the fluorescence is the intrinsic fluorescence, and (2) in a polar solvent (propylene glycol), where the fluorescence is from the indole-alcohol exciplex. Dual-level emission is expected for the intrinsic fluorescence but not for the exciplex. The measurements consist in determining the fundamental polarization (which is the maximum attainable value) of the fluorescence for different exciting wavelengths. This is achieved by working in rigid media at 220°K. The value of polarization at a given wavelength of excitation is the sum of the products of the fractional absorption at this wavelength of a given oscillator and a function of the angle this oscillator has to the emitting oscillator. The lowest absorption band of indole contains two electronic transitions, ^1L_a and ^1L_b, which have a large angle between them. In propylene glycol the polarization spectrum, though complex and with fine structural details, is moderately polarized over the lowest absorption band (20–35%). The spectrum can be interpreted on the basis of two absorptions and a single emitting level. In mineral oil a different behavior is found; now the polarization spectrum is hardly polarized (2%), has no fine-structural details, and can only be interpreted by assuming that the emission is from two levels that are virtually perpendicular to each other.

In conclusion, the polarization spectra of indole in polar and nonpolar solvents are markedly different and are consistent with the idea of dual-level emission in the hydrocarbon solvent.

Reference

[1] Research supported jointly by the National Cancer Institute and the U.S. Atomic Energy Commission under contract with Union Carbide Corporation.

2.36 OPTICAL PROPERTIES AND CONFORMATIONS OF POLYNUCLEOTIDES

J. A. Knopp J. W. Longworth R. O. Rahn

By dissolving lyophilized fibers of polynucleotides, such as DNA, tRNA, and $r(A)_n$, a random coiling conformation has been obtained. When this technique was applied to polyriboguanylic acid, $r(G)_n$, an unknown conformation was obtained. According to the

criteria of extinction coefficient and circular dichroism, this conformation has a high degree of order, but temperature melting experiments have been unsuccessful in obtaining any melting of $r(G)_n$ in this solvent up to temperatures of 120°C. Temperature melting experiments of $r(G)_n$ in a mixed solvent of water and ethylene glycol suggest that there is a high degree of affinity of $r(G)_n$ for ethylene glycol. Furthermore, discrepancies have been found between different sources of poly $r(G)_n$ in the temperature melting curves in water. At the present time the conflicting characteristics of this polynucleotide cannot be resolved.

2.37 MECHANISM OF INACTIVATION OF TRANSFORMING DNA BY X RAYS

M. L. Randolph Jane K. Setlow

Physical integration of transforming DNA into the DNA of the recipient cell is essential in genetic transformation of bacterial cells. Competent *Haemophilus influenzae* cells that integrate transforming DNA release equivalent amounts of their own DNA which can be measured as the release of radioactivity from cells containing labeled DNA.[1] We have used this method to compare the loss of integration of x-irradiated transforming DNA with the loss of its transforming ability in wild-type and three uv-sensitive strains of *Haemophilus*. The transforming DNA was exposed to x rays either in phosphate buffer (for determination of the indirect effect of the irradiation) or in 10% yeast extract (direct effect). The radiosensitivity in buffer is about 100 times that in yeast extract. For all strains the dose curve for loss of integration by direct and indirect effects is similar to the loss of transforming ability of the least-sensitive drug marker in the same irradiated DNA. These results indicate that the principal biological effect of x irradiation on transforming DNA is reduction of integration into the host genome, whereas with uv irradiation loss of integration makes almost no contribution to biological inactivation of transforming DNA.[2] For the direct effect of x rays the number of single-strand breaks produced per strand of transforming DNA by the $1/e$ dose for inactivation of transformation (about 0.4 mr) is roughly estimated as 10, and hence there is perhaps one double-strand break per strand at this dose. Compared with the other strains, strain DB116 is less efficient in mending single-strand breaks, and the radiosensitivity of transforming DNA is somewhat greater when assayed on DB116.[3] Hence our results are consistent with the notion that double-strand breaks induced by x irradiation in DNA make an important contribution to loss of transforming ability, whereas single-strand breaks are significant only when cells repair them slowly.

References

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2.38 DEGRADATION KINETICS OF THE DNA OF *HAEMOPHILUS INFLUENZAE* AFTER X IRRADIATION

M. L. Randolph

Analysis of the kinetics of degradation of DNA after x irradiation (or other cellular insult) offers a means of testing models of in vivo molecular processes – in particular, regarding the nature and rate of the responsible enzymatic process and the possibility of degradation ending either by a potentially first step of repair or by the presence of unique degradation-resistant sequences in the DNA.

Haemophilus influenzae cells were labeled with ^{3}H-thymidine while growing to log phase and were x irradiated and incubated in growth medium for various durations. Enzymatic degradation of their DNA, as measured by acid-insoluble radioactivity, decreased with time to dose-dependent plateau values. Irradiated cells with ^{3}H-labeled DNA and unirradiated cells with ^{14}C-labeled DNA were lysed onto alkaline sucrose gradients. The radiation-induced breaks per single strand are calculated from estimates of differences in number-average molecular weights. Assuming that single-strand breaks occur at random and that newly synthesized DNA lengths are distributed as are ages of log-phase cells, I derived formulas giving the DNA remaining as a function of time for the eight models resulting from the options circular or linear DNA, unilateral or bilateral degradation from each break, and degradation stopped only by ends of strands or stopped at random times and by ends of strands. The observed plateaus exclude the models of unhindered degradation for circular DNA and for linear DNA with bilateral degradation. The magnitudes of plateaus exclude linear DNA with unhindered unilateral degradation. Hence degradation often stops before reaching the end of a strand. This is a necessary, but not sufficient, condition for repair. Best-fit coefficients and coefficients of variance are being obtained by computer programs with

the Division's time-share computer facilities. Preliminary results favor linear DNA with unilateral degradation which has a random probability of stopping.

The formulation, although here tested only with *Haemophilus* after x irradiation, is of a general nature and should be equally applicable to other cells and to treatments with other radiations or chemicals.

2.39 A QUALITATIVE INTERPRETATION OF RBE DATA

M. L. Randolph

Biological radiosensitivity may be interpreted in terms of Rossi's $Z(r)$ factor, which is the energy deposited per unit mass by ionizing radiation in a sphere of radius r. One may consider $Z(r)$ as a microscopic concentration of energy deposition. Our interpretation is for effects whose expression requires only a minimum energy deposition within a critical volume. This is a theory of indirect action without dose-rate dependence, repair, or anoxia. Under these assumptions, radiosensitivity is zero if $Z(r)$ is less than the critical value $Z_0(r)$ and varies as $Z_0(r)/Z(r)$ at greater $Z(r)$.

Measurements of $Z(r)$ distributions, $D(Z)$, show that in certain cases the average $Z(r)$, $\bar{Z}(r)$, for low-LET radiation (^{60}Co) increased over a dose range, while that for high LET (1-MeV neutrons) remains constant.[1] This reflects that for equal doses there are many more tracks for ^{60}Co than for neutrons; thus over some low dose range increasing the ^{60}Co dose increases the tracks per sphere and $\bar{Z}(r)$, whereas increasing the neutron dose merely increases the number of spheres containing a track. At very high doses $\bar{Z}(r)$ approaches the macroscopic value of dose for all radiations.

The biological effect of radiation absorbed at energy concentration $Z(r)$ is the dose times the radiosensitivity at that concentration, and the observed effect is the sum of effects at all concentrations. Thus observed effects depend on system parameters $Z_0(r)$ and r and physically controllabe variables, dose and $D(Z)$. Theoretically one might determine $Z_0(r)$ and r from $D(Z)$ measurements and a single wide dose curve.

Consider expected results for two extreme $D(Z)$ cases. First, let the LET be low and $Z_0(r)$ and r such that a single track crossing the sphere seldom deposits $Z_0(r)$. At low doses, where single traversals predominate, $\bar{Z}(r)$ and hence radiosensitivity are constant. At doses where multiple traversals occur, $\bar{Z}(r)$ increases, reaches $Z_0(r)$, and finally approaches the numerical value of dose. Simultaneously, the radiosensitivity increases to a maximum where $\bar{Z}(r) \simeq Z_0(r)$. Next, for the same $Z_0(r)$ and r let the LET be high so that most traversals deposit more than $Z_0(r)$. Now $\bar{Z}(r)$ and radiosensitivity are constant to much greater dose. When multiple traversals occur, $\bar{Z}(r)$ increases and radiosensitivity decreases (see Fig. 2.39.1).

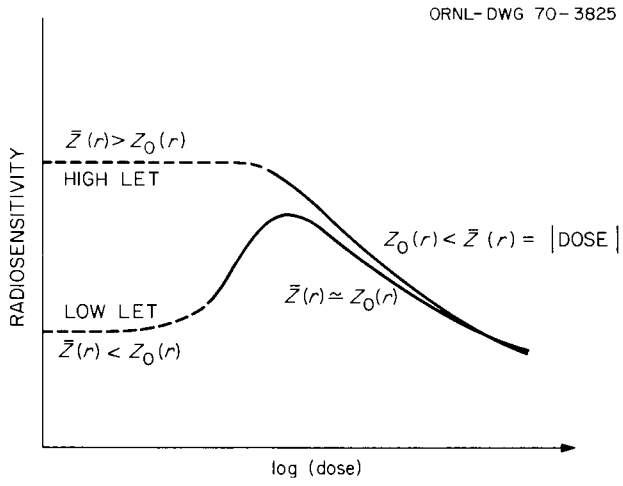

Fig. 2.39.1 Theoretical Variation of Radiosensitivity with Dose for Low and High LET Radiations.

Predictions from this theory include:

1. Statistical determinations of parameters for dose curves obtained from biological data may well show odd dependency, such as $(\text{dose})^{1.7}$, depending on the dose range.
2. For all radiations linear dose curves obtain at very low doses, and the limit of linearity increases with LET.
3. At very high doses the radiosensitivity of a given system to all radiations becomes equal.
4. RBE will often depend on dose range and hence may change with dose.

Reference

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2.40 LUMINESCENCE OF MERCURIATED DNA – THE HEAVY ATOM EFFECT

R. O. Rahn Maria Del Carmen Battista

Mercuric ions (Hg^{2+}) bind directly to the purine and pyrimidine bases in nucleic acids and consequently cause large changes in the physical and optical properties. It was of interest, therefore, to examine the

effect of Hg^{2+} binding on the emission properties (phosphorescence and fluorescence) at 77°K of various homo- and heteropolymers. In all the polymers studied the fluorescence is quenched by Hg^{2+}, and the phosphorescence lifetimes are shortened. These results have been interpreted in terms of the heavy-atom effect and are the first demonstration of this effect in nucleic acids. (In this effect, the heavy atom, when bound to a molecule undergoing light absorption, increases the probability of a transition between the singlet and triplet states.) Furthermore, in poly dT and poly C, Hg^{2+} binding enhances the phosphorescence by an order of magnitude, while in poly A and poly G, Hg^{2+} quenches the phosphorescence by an order of magnitude. Hence, low-temperature phosphorescence in conjunction with Hg^{2+} binding is a very sensitive way of distinguishing between purine and pyrimidine polynucleotides.

In thymine-containing heteropolymers such as DNA and poly dAT, where the binding of Hg^{2+} is presumed to occur initially at the thymine residues, an enhancement of the phosphorescence occurs at low Hg^{2+} concentrations, that is, for $r < 0.25$, when only the thymine residues are mercuriated. At higher concentration of Hg^{2+}, bases other than thymine become complexed with Hg^{2+}, and the phosphorescence is quenched. We have interpreted these results in terms of singlet energy transfer from thymine:Hg^{2+} to mercuric complexes of the other bases. The absorption spectra of A, G, and C indicate that these bases when mercuriated have lower-lying singlet levels than thymine:Hg^{2+} and, therefore, can act as energy sinks. Transfer to either A or G would result in the phosphorescence being quenched since these bases do not emit when mercuriated.

We have found that in a series of DNA's, varying in AT/GC ratio, the relative amount of Hg^{2+} necessary to achieve a maximum phosphorescence intensity is proportional to the thymine content of the DNA. This result may provide a basis for a simple spectroscopic technique for measuring the AT/GC ratio of a variety of DNA's.

2.41 THE INFLUENCE OF MERCURIC ION ON THE PHOTOCHEMISTRY OF NUCLEIC ACIDS – SINGLET ENERGY TRANSFER IN DNA

R. O. Rahn L. C. Landry

The quantum yield of uv-induced dimer formation in TpT and poly dT is not affected by Hg^{2+} binding. In *E. coli* DNA no quenching of the dimerization reaction occurs at levels of Hg^{2+} such that only the thymine residues are mercuriated ($r < 0.25$). In fact, Hg^{2+} binding seems to increase the rate of dimerization below $r = 0.25$. However, when all the bases in DNA have Hg^{2+} associated with them ($r = 1$), then the rate of dimerization and the photosteady concentration of dimers are greatly reduced. We believe that this quenching is due to singlet energy transfer from the mercuriated thymine complexes to the mercuric ion complexes of the other bases, which, because of their lower-lying singlet levels, act as energy traps. This energy transfer process competes with both the dimerization reaction and intersystem crossover for the excitation energy. Hence, less of the absorbed energy is available for photochemistry, and a slower dimerization rate results. This mechanism was used in Sect. 2.41 to explain the quenching of the phosphorescence of DNA:Hg complexes.

The action spectrum for dimer formation in the completely mercuriated DNA indicates that dimers cannot be made by exciting at the long-wavelength edge of the absorption spectrum. Since Hg^{2+} shifts the absorbance of all the bases except thymine to long wavelengths, we conclude that thymine dimers are formed in mercuriated DNA following absorption by thymine only and that no transfer of energy to thymine from the mercuriated complexes of the other bases occurs.

Mercuriation had no effect on the rate of dimer production in DNA when acetophenone sensitization of the triplet state was employed. Hence the triplet state of thymine:Hg^{2+} is assumed to lie lower than that of the Hg^{2+} complexes of the other bases.

The steady-state concentration of thymine dimer obtained at a fixed wavelength of irradiation is determined by the relative rate of dimer formation and dimer splitting. Photoreversal has been used in the past to demonstrate the role played by dimers in the biological inactivation of transforming DNA. Dimers have been photoreversed by irradiation either at shorter wavelengths or in the presence of proflavin. We have found that the steady-state concentration of dimers in DNA for 254-nm irradiation is reduced sevenfold upon adding Hg^{2+} and continuing the irradiation.

The relative ease and the specificity with which Hg^{2+} interacts with DNA, together with the pronounced effect of Hg^{2+} binding on the photochemistry of DNA, suggest a possible use for this ion in probing the accessibility and the environment of DNA in cells.

2.42 REDUCTION OF THE UV SENSITIVITY OF TRANSFORMING DNA AND PHAGE BY MERCURIC IONS

R. O. Rahn Jane K. Setlow S. P. Leibo

We have shown that the rate of thymine dimer formation in DNA is reduced when mercuric ions (Hg^{2+}) are bound. Furthermore, dimer reversal occurs when Hg^{2+} is added to previously irradiated DNA and the DNA is subjected to further irradiation at the same wavelength. Since thymine dimers cause biological inactivation, it was of interest to see whether Hg^{2+} would reduce the uv-induced inactivation of transforming DNA and phage.

Haemophilus influenzae transforming DNA was irradiated with and without bound Hg^{2+}. The rate of biological inactivation as measured by the loss of transforming ability was ~3 times less with Hg^{2+} bound. Furthermore, the biological activity increased when Hg^{2+} was added to previously irradiated transforming DNA and the irradiation continued; that is, reversal of the dimers occurred, leading to an increase in the transforming ability.

Phage T_4D was also irradiated with and without Hg^{2+}. We assume on the basis of optical absorbance and circular dichroism measurements that Hg^{2+} binds to the DNA of phage in the same way as it does to isolated DNA. Treatment of the mercuriated phage with either Cl^- or CN^- complexes the Hg^{2+}, and the biological activity of the phage so treated is not impaired. The sensitivity of the phage to uv was two to three times less with Hg^{2+} bound.

The reversible binding of Hg^{2+} to DNA makes this ion a convenient probe for investigating the photobiology of viruses and, possibly, whole cells such as bacteria.

2.43 THYMINE DIMER FORMATION IN POLY d(TppT) AND IN APURINIC ACID

R. O. Rahn L. C. Landry

The photoinduced thymine dimer is readily formed in the systems containing thymine residues coupled via a sugar-phosphate linkage such as TpT and poly dT. Dimers are also formed in the dinucleotide TppT, but the yields are 40% lower than in TpT. We have measured dimer formation in a polymer containing TppT residues which was prepared from poly d(A-T) by depurination. No dimers are formed in poly d(A-T) because adenine residues separate the thymines. However, removal of adenine leaves a polymer, poly d(TppT), in which dimers are formed about five times slower than in poly dT. The formation of dimers in such a system helps to explain the high dimer yields observed previously in apurinic acid, since depurination of DNA results in thymine normally separated by either adenine or guanine becoming adjacent and therefore dimerizable.

Chromatographic analysis of poly d(TppT) irradiated at $\lambda > 300$ nm in the presence of acetophenone shows that two thymine dimers, the cis-syn dimer $\hat{TT}_1$ and the trans-syn dimer $\hat{TT}_2$ are formed in the ratio $\hat{TT}_1 : \hat{TT}_2$ of 5:1. This same ratio was obtained for TpT, apurinic acid, and highly denatured DNA. However, very little $\hat{TT}_2$ was formed in ordered systems such as DNA or poly dAdT.

Treatment of irradiated poly d(TppT) with the photoreactivating enzyme did not result in any monomerization of dimers. Also, no evidence was obtained, using optical techniques, for the interaction between poly d(TppT) and poly A. We conclude that from these results that the bases in poly d(TppT) are not stacked in such a way as to mimic poly dT.

The concentration of thymine dimers in apurinic acid following a saturating dose of direct irradiation, either at 254 or 280 nm, was twice that obtained for native DNA. Furthermore, photosensitization of apurinic acid with acetophenone gave 52% thymine dimer as opposed to 38% in native DNA. From these increases in dimer yield upon depurination, we conclude that dimerization occurs in apurinic acid between thymines which in native DNA are normally separated by purine residues.

2.44 PHOTOCHEMISTRY OF PYRIMIDINES IN ICE

R. O. Rahn K. H. Walbert[1]

We have been studying the various pyrimidine photoproducts which are formed upon irradiation of frozen water solutions of the pyrimidines. In particular, we have compared the formation of the adduct and trimer in thymine and uracil. These two photoproducts are easy to observe using absorbance spectroscopy, since (1) the adduct absorbs at 315 nm and (2) the trimer is converted into the adduct upon irradiation in solution.

The results presented in Table 2.44.1 show that adduct and trimer formation in uracil occurs about three times less efficiently than in thymine. However, the amount of dimer formed is about the same for both uracil and thymine.

It is difficult to speculate at this point as to whether the difference in the yields of adduct and trimer between uracil and thymine are due to variations in crystalline properties or whether the methyl group has some effect. The small yields of photoproduct in uracil,

Table 2.44.1. Comparison of Photoproduct Yields in Thymine and Uracil

Photoproduct	Yield (%)	
	In Thymine	In Uracil
Cyclobutane dimer	65	60
Hydrate		1
Adduct	3	1
Trimer	10	3.7

coupled with the poor chromatographic separation of the adduct and the trimer, complicate any attempt to further our knowledge about the uracil photochemistry.

Reference

[1] Student trainee.

2.45 PTERIDINES AND PHOTOSYNTHETIC REACTION CENTERS

N. A. Nugent Nicholas Rigopoulos
R. C. Fuller

Extraction of pteridines at pH 9 from *Rhodospirillum rubrum* pigment protein results in changes in the far-red (700–1000 nm) absorption spectrum attributable to loss of the reaction center complex. Since the extraction method does not remove any chlorophyll, the loss of the reaction center must be due to extraction of one or more small molecules specifically associated with the reaction center.

Addition of tetrahydropteridine to pigment protein which has lost its reaction-center absorption bands causes a regeneration of these characteristics. Such a phenomenon strongly suggests that the absorption bands associated with the reaction center are the result of the formation of a specific complex between chlorophyll and pteridine. Similar results with *Euglena* chloroplasts and the green bacterium *Chloropseudomonas ethylicum* suggest a wide occurrence of this type of complex in photosynthetic systems.

Model complexes involving pteridine, flavin, iron, ferredoxin, and chlorophyll protein are currently under investigation. The molecular complex tetrahydropterin-Fe^{3+}-flavin exhibits electron transport properties. Specifically, an electron is passed from the pterin to the flavin ring, resulting in the appearance of the flavin radical with an absorption maximum at 550 nm. The Fe^{3+} is essential for this transfer. Reduced pterins and flavins form chelate-type compounds, with iron, which then can undergo oxidation or reduction. The iron of ferredoxin can be chelated in a similar manner, creating a protein-bound electron transport complex. This type of "multimolecular" complex is being investigated in greater detail.

An additional argument favoring the involvement of pteridines in photosynthetic electron transport is the effect of dihydropteridine on the intensity of the delayed fluorescence. A fourfold enhancement was observed when dihydropteridine was added to a suspension of spinach chloroplasts. The pteridine analog 2,4-diamino-5,6,7,8-tetrahydroquinazoline, on the other hand, was found to diminish the intensity of the delayed emission to a value one-half that of the control.

These experiments further strengthen the idea that pteridine is involved in the early events of photosynthetic electron transport.

2.46 EFFECTS OF CHLORAMPHENICOL AND CYCLOHEXIMIDE ON AMINO ACID INCORPORATING SYSTEMS OF CHLOROPLASTS FROM WHEAT, PEA, AND *EUGLENA*[1]

R. M. Smillie[2] Bonnie J. Reger
Nicholas Rigopoulos

Considerable knowledge has been acquired on the role of chloroplast DNA and chloroplast ribosomes in the biogenesis of the chloroplast. Smillie has employed the antibiotics chloramphenicol (CAP) and cycloheximide (CH) with *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 (70s), and (2) chloroplast DNA codes for chloroplast ribosomal RNA. CAP preferentially inhibits 70s ribosomes, whereas CH inhibits 80s ribosomes. We have now obtained in vitro concentration curves for the effect of CAP and CH on ribosomal systems from proplastids and chloroplasts of *Euglena*, wheat, and pea. Attempts to obtain like information from the cytoplasmic systems have not been successful.

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 contaminants. Platings of reaction mixtures were done

Table 2.46.1. Percent Inhibition of Plastid Protein Synthesis by Chloramphenicol (CAP) and Cycloheximide (CH)

	CAP							CH				
	5 μg/ml	10 μg/ml	20 μg/ml	40 μg/ml	80 μg/ml	200 μg/ml	800 μg/ml	1 μg/ml	5 μg/ml	20 μg/ml	200 μg/ml	400 μg/ml
Wheat (green)	30	50	59	69	72	79	85	2	6	11	11	
Pea (green)	49	59	71	80	83	86	90	16	14	22	22	19
Euglena (green)	46		78	86		24			9		9	

routinely, counts of chloroplasts to mitochondria were made, and activities were determined. Plastid preparations from young wheat leaves contained as few as one bacterium per 10^6 plastids. Counts of actual number of particles smaller than chloroplasts in preparations of wheat plastids yielded one particle (heavy mitochondria, chloroplast fragment, or peroxisome) per 1.3 to 2.0 intact plastid. By comparing the amino acid incorporating activity of the 1000 × *g* fraction with that of a fraction obtained by centrifugation at a higher speed, and by estimating the relative numbers of mitochondria and peroxisomes in the two fractions on the basis of the NAD–malate dehydrogenase activity, it was estimated that these organelles could contribute no more than a few percent to the amino acid incorporating activity of the 1000 × *g* fraction.

The incorporating ability of proplastids from pea and *Euglena* was essentially negligible, whereas the proplastids from wheat essentially equaled the incorporation obtained from chloroplasts. Proplastids from wheat were affected in the same manner as chloroplasts by CAP and CH. Amino acid incorporating abilities were expressed as a rate (counts per minute) on an RNA basis. Typical results of the effect of CAP and CH can be seen in Table 2.46.1.

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[2] Plant Physiology Unit, Division of Food Preservation, CISIRO; Dept. of Botany, University of Sydney, Sydney, Australia.

2.47 FRUCTOSE-1,6-DIPHOSPHATASE OF *RHODOSPIRILLUM RUBRUM*

I. R. Joint Nicholas Rigopoulos R. C. Fuller

Carbon dioxide is fixed via the Calvin cycle in autotrophically grown *Rhodospirillum rubrum.*[1] In higher plants it has been postulated that the Calvin cycle is controlled by the diffusion of intermediates of the cycle – notably fructose-1,6-diphosphate, sedoheptulose-1,7-diphosphate, and ribose-5-phosphate – out of the chloroplast.[2] The diffusion clearly cannot be the means of regulation of the Calvin cycle in photosynthetic bacteria, which have no chloroplasts. The cycle in these organisms could only be regulated by inhibition of the enzymes, and the enzymes most likely to be important control points are those which catalyze thermodynamically irreversible reactions. Such enzymes of the Calvin cycle are ribulose-1,5-diphosphate carboxylase, fructose-1,6-diphosphatase, sedoheptulose-1,7-diphosphatase, and ribulose-5-phosphate kinase. We are attempting to purify the fructose-1,6-diphosphatase in order to study the effect of various metabolites on the activity of the enzyme in the hope of suggesting a mechanism of control of the Calvin cycle. Experiments so far have shown that fructose diphosphatase increases in activity when *R. rubrum* is transferred from heterotrophic to autotrophic growth conditions. The activity also depends on the age of the autotrophic culture. Highest levels of fructose diphosphatase were found during logarithmic growth, and the level of fructose diphosphatase fell dramatically as stationary phase was approached.

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2.48 PHOTOSYNTHESIS

W. A. Arnold J. R. Azzi S. F. Carson
J. B. Davidson[1]

Photosynthesis, by green plants, is the process by which the carbon dioxide put in the atmosphere by the automobile is removed and replaced by oxygen. We are studying the delayed light and fluorescence of green plants to learn about the initial steps in the mechanism.

Action Spectra for Delayed Light. – In 1951 a communication from this laboratory described a light emission by green plants which lasted for some seconds after illumination.[2] Since that time the delayed light has been under investigation in the hope that it will lead to a better understanding of the first steps in photosynthesis.

The action spectrum of the delayed light is basic to any understanding of the phenomenon. Although several action spectra have been published, the discovery by Jones[3] that the delayed light is a strictly linear function with respect to the energy of a short flash of light (providing that the plants were previously kept in dim light for some minutes) and the development of a convenient radiometer by the Yellow Springs Instrument Company make it possible to take very much better action spectra. Using these criteria we have determined action spectra for green, blue-green, and red algae and photosynthetic mutants. The data are being assembled for publication.

Optical Cross Section of the Photosynthetic Unit. – In our first report on glow curves,[4] we estimated the optical cross section for the unit that stores energy to be larger than 110 chlorophyll molecules. The size of the unit could not be determined accurately due to scattering of light by ice crystals in the frozen sample. Since then we have found that chloroplasts suspended in 66% glycerol, freezing point –47°C, will make perfectly satisfactory glow curves, thus avoiding ice crystals when making glow curves at –30°C. Under these conditions we have determined the optical cross section for two of the three peaks in the glow curves. These data show one peak to correspond to 230 chlorophyll molecules and the other to 150 chlorophyll molecules.

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2.49 BETA-PARTICLE-EXCITED FLUORESCENCE DECAY INSTRUMENT

R. M. Pearlstein W. A. Gibson

The past year saw significant progress in the development of the instrument to determine fluorescence decay profiles of chloroplasts and other biological 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. Using an RCA C70045C as the second photomultiplier, we achieved a time resolution of 330 psec in 1968 which we reduced to 270 psec on a routine basis in 1969.

During 1969, the instrument was calibrated for the first time with aqueous solutions of fluorescein at various values of pH. At high pH, for which fluorescence lifetimes of fluorescein are well known, we observed a lifetime of 4.6 nsec, in excellent agreement with values reported in the literature. At lower pH, we observed, possibly for the first time anywhere, biphasic decay due to roughly equimolar amounts of protonated and unprotonated fluorescein. Unfortunately, the fluorescein tests also confirmed that the C70045C photomultiplier provides too low a signal-to-noise ratio for use in observing fluorescence from chloroplasts. Therefore we installed an RCA C31000F photomultiplier, which is a fast tube with an extended-red multialkali photocathode and which just became available in 1969. When the C31000F is cooled to dry-ice temperature, it has a signal-to-noise ratio for chloroplast fluorescence several hundred times that of the C70045C. With the C31000F, we have recently observed the unambiguous

decay of fluorescence from chloroplasts excited by beta particles. The only shortcoming of the C31000F is that so far it has yielded a time resolution no less than 700 psec. Even so, we expect to achieve the major objectives of our study of chloroplasts with the instrument during 1970.

As in the past, we gratefully acknowledge the cooperation of the Neutron Physics Division, particularly the help of N. Hill, who is on loan to that Division from the Instrumentation and Controls Division.

2.50 EXCITON QUENCHING IN LINEAR POLYMERS AND PHOTOSYNTHETIC UNITS

R. M. Pearlstein R. P. Hemenger[1]

When the energy of visible, uv, or ionizing radiation is absorbed by an interacting group of identical molecules, mobile excited states (excitons) are produced. These excitons sometimes can be quenched, that is, removed from the interacting molecular group, by a second class of molecules present in much smaller concentration, which then possesses some fraction of the excitation energy. Examples of biological systems in which such processes are important include photosynthetic units, in which the two classes of molecules are bulk chlorophyll and reaction-center chlorophyll respectively; DNA with intercalated dye, in which the two are the nitrogenous bases and the dye; polynucleotides, in which the sites of thymine-dimer formation become quenchers; and fluorescent-dye-labeled antibody containing aromatic amino acids. The influence of the exciton motion prior to quenching on the quenching process itself is, in most cases, poorly understood in detail. We are attempting to acquire an understanding of this influence by theoretically comparing the effects on quenching, as might be observed in fluorescence kinetics, of two extreme types of excitons.

During the past year, a study by one of us (R. M. P.) on the comparative kinetics of these two exciton types under weak quenching in linear polymers was completed, and it is being readied for publication. Some of the results of this study were mentioned in the 1968 report.[2] Work undertaken in 1969 extends the earlier study in two ways. First, the kinetics of strongly coupled or coherent excitons in linear polymers were derived for an arbitrarily strong terminal quencher, with a surprising result. Whenever the number of identical molecules in the absorbing group is two or more per quencher, the effectiveness of the quencher begins to diminish above a certain optimum quenching strength. In other words, there is a "best" coupling between the quencher and the quenched. Second, some calculations have been done on the weak quenching of coherent excitons in square lattices; these should be applicable to photosynthetic units. The results so far indicate profound differences in the behavior of the two types of excitons in such lattices. Also during 1969, one of us (R. M. P.) showed that our calculations on linear polymers can be applied to energy transfer in polynucleotides. During 1970, we hope to extend our results to cover the case of partially coherent excitons, which are probably the most prevalent in nature.

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2.51 RBE OF FAST NEUTRONS IN GROWTH INHIBITION: DIRECT EVIDENCE IN WHEAT FOR A COMPONENT REQUIRING CELL DIVISION

A. H. Haber L. L. Triplett F. F. Haywood[1]
D. R. Ward[2]

It is well known that neutrons (and other "densely ionizing" radiations) are more effective than gamma rays (and other "sparsely ionizing" radiations) for producing damage as measured either by cytogenetic or by growth criteria. We here report first on the RBE of fast neutrons (relative to gamma rays) in reducing growth in a typical system in which the growth inhibition can be influenced by two typical cytogenetic radiation effects: (*a*) mitotic inhibition and (*b*) nuclear imbalance resulting from division of cells having chromosomal aberrations. This RBE is then compared with values of RBE in three systems in which the two aforementioned cytogenetic radiation effects play no role in radiation injury. In two of these systems, cell growth occurs without cell division, and in a third system cell death occurs in absence of both cell growth and cell division.

In all cases we compare fast neutrons from the Health Physics Research Reactor and gamma rays from a ^{60}Co source. Neutron dosimetry was afforded by measurements of the induced radioactivity in standard sulfur pellets.[3] Neutron dose rates ranged from 0.1 to 1 kilorad/min, and the reactor was operated at 100 w or 2 kw. The gamma-ray dose rates were about 5.5 kilorads/min. In each system the RBE was calculated as the ratio of gamma-ray dose to neutron dose necessary to give each of several selected values of measurable injury.

Within the dose range applied to each of the four systems, the RBE so calculated was largely independent of dose or degree of measurable damage. The biological systems, the range of doses used, and the calculated values of RBE are given below.

A. **Typical System in Which Cell Division Can Contribute to Damage.** – Young wheat seedlings were irradiated with 200 to 2000 rads when their coleoptiles were 3 to 4 mm long. RBE was determined for reduction of final size of the first foliage leaf, which final size was attained with cell divisions after all the doses used. Internally consistent values of RBE (6.7 and 6.7) were found in each of two independent experiments.

B. Seedling Irradiation of Gamma Plantlets. – Gamma plantlets are seedlings growing with a high degree of physiological normalcy despite absence of cell division due to gamma irradiation of the dry grain.[4] After dry seed irradiation with 500 kilorads of gamma rays, the growing gamma-plantlet *seedlings* were irradiated with 100 to 600 kilorads of neutrons or gamma rays when their coleoptiles were 3 to 4 mm long. Leaf length was measured as in system A. Internally consistent values of RBE (2.5 and 2.3) were found in each of two independent experiments.

C. Plants from Unirradiated Grain but Still Lacking Cell Division After Irradiation. – The seedlings were selected and irradiated as in system A, except that higher doses (100 to 600 kilorads) were used, and measurements of leaf growth were made only three days later. In this system there was little or no cell division between the times of irradiation and leaf measurement, as shown by both morphologic and growth studies of irradiated plants.[5] Internally consistent values of RBE (2.2 and 2.1) were found in each of two independent experiments.

D. Tissue Death in Absence of Both Cell Growth and Cell Division. – Light-induced photodestruction of chlorophyll is a general index of ionizing-radiation-induced death in green wheat leaf tissue.[6] This criterion was used to study actions of 100 to 700 kilorads on excised 3-cm leaf tips from seven-day-old plants. The leaf tips were exposed to 1000 ft-c of white light for three days. Internally consistent values of RBE (2.9 and 2.9) were found in each of two independent experiments.

In system A the radiation injury expressed as growth inhibition can be considered to result in part from the two cytogenetic effects of (*a*) mitotic inhibition and (*b*) nuclear imbalance resulting from division of cells having chromosomal aberrations; both these effects involve cell division. In addition, other physiological effects can contribute to inhibition of growth and its RBE in system A. In system B, however, the two cytogenetic effects cannot occur, because the gamma plantlets have no cell divisions at the time of seedling irradiation. System C differs from B in that the unirradiated seedling controls do grow with cell divisions. The cytogenetic effects, however, cannot contribute to RBE in system C, because mitosis was essentially prevented by the radiation doses used. Thus the RBE values for systems B and C presumably reflect only the RBE for physiological damage leading to growth inhibition apart from the cytogenetic effects. System D is not strictly comparable with the other three systems, since death in nongrowing cells, rather than growth inhibition, is studied. Nevertheless, the RBE for system D resembles that in B and C, in which cell division is also incapable of contributing to the RBE of neutrons. These findings indicate that the occurrence of cell division and consequent cytogenetic radiation effects, *a* and/or *b*, contribute to the high RBE of neutrons in growth inhibition and cell death. In absence of these radiation effects requiring cell division, the RBE of neutrons is considerably decreased.

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2.52 OPPOSITE GROWTH RESPONSES OF STEMS VS LEAVES TO GAMMA IRRADIATION OF WHEAT SEEDLINGS

D. E. Foard Rhonda F. Irwin

A dose of 25 kilorads stimulated growth of the first foliage leaf, but not of the stem, of wheat gamma plantlets (seedlings growing without cell division after heavy gamma irradiation of grain).[1] We now report a promotion of stem growth by doses of radiation that inhibit growth of the first leaf of wheat seedlings grown from unirradiated grain; these seedlings are growing with cell divisions at the time of irradiation. Stem length of seedlings at the time of irradiation was 532 ± 104 μ (mean and 95% confidence limits). Irradiation

doses were 25, 50, or 100 kilorads. Eleven days after irradiation, when growth of the shoots had ceased in the irradiated seedlings, stem length of unirradiated control seedlings was 1526 ± 81 μ. Stem lengths of irradiated seedlings were 1679 ± 111, 1946 ± 120, and 1977 ± 82 μ after 25, 50, and 100 kilorads respectively. In a second experiment these unexpected results were repeated. Stem lengths of unirradiated seedlings were 1690 ± 121 μ, whereas stem lengths of irradiated seedlings were 1897 ± 228, 1946 ± 86, 2121 ± 150, 2197 ± 122, and 2141 ± 178 μ after 20, 40, 60, 80, and 100 kilorads respectively. There is no evidence of continued cell division after irradiation, and anatomically these stems resemble those of gamma plantlets.[2] Presumably, stem lengths of unirradiated control seedlings would eventually exceed those of irradiated seedlings; however, during the 14-day period observed, gamma radiation promoted growth in length of the stem. Final leaf length of unirradiated seedlings was approximately 13.6 cm, whereas final leaf length of irradiated seedlings was approximately 3.5 cm after 20, 40, 60, 80, and 100 kilorads. Thus doses of 20 to 100 kilorads to these seedlings promote growth in length of stems to varying degrees, but inhibit growth of the first foliage leaf to the same degree in all cases. Therefore it is not likely that this is an example of a compensatory growth phenomenon. Rather the differences in growth of the two organs probably reflect inherent differences in response to ionizing radiations.

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2.53 STUDIES OF CHLOROPLAST SENESCENCE IN MATURE LEAF TISSUE

A. H. Haber Patricia J. Pate[1] Paula J. Thompson[2]

In the apical portion of wheat leaves, senescence occurs without cell growth and division, two processes which complicate studies of animal senescence. The disintegration of chloroplasts can be followed uncomplicated by any chloroplast formation either by keeping the material in darkness or by treating with aminotriazole in the light.[3] We here report on three questions we have approached using this material.

Independent Actions of Cytokinin and Light in Retarding Senescence. – When aminotriazole was used to prevent chloroplast formation, we determined the optimal concentration (10^{-5} *M*) of kinetin and the optimal intensity (350 μw/cm^2) of white light for retarding senescence. The effectiveness of kinetin was comparable in darkness and in tissue exposed to light intensities saturating for senescence retardation. Similarly, illumination was comparably effective in retarding senescence of tissue treated with kinetin and of tissue not treated with kinetin. These findings suggest that the regulations of senescence by cytokinin and by light involve largely different mechanisms. The results should be useful in providing additional experimental controls for certain studies concerned with the role of cytokinins in regulating senescence.

Age and Rate of Senescence. – Minot suggested that the *rate* of animal senescence decreases with age, whereas Needham[4] suggested that it increases with age.[4] With these two opposing points of view in mind, we studied the rate of chloroplast senescence in darkness in developmentally comparable tissue of the first foliage leaf in previously light-grown plants of different ages. We found no evidence for significant differences in rate of chloroplast senescence for plants whose ages ranged from 6 to 18 days at the time they were transferred to continuous darkness. Since this time interval spans the greater part of the period of development and senescence of the first foliage leaf, we conclude that neither Minot's nor Needham's theory of senescence applies to our system.

Temperature Dependence of Senescence. – From studies of specific mortality rates of invertebrates, Strehler[5] expressed the relation between rate of senescence and temperature as an activation energy of 18,400 kcal. Since calculation of an activation energy may not be theoretically justifiable, we consider his data as showing a Q_{10} of approximately 2.9 for rate of senescence in the temperature range (10–30°C) studied. We placed seven-day-old wheat seedlings in continuous darkness at 10, 15, 20, 25, and 30°C and followed chloroplast senescence in the nongrowing apical part of the first leaf of intact plants. We calculated the Q_{10} from the times necessary at each temperature to produce the same arbitrary level of chlorophyll loss. Several arbitrary levels of chlorophyll loss were used. We found a consistent and repeatable value of 1.7 for this Q_{10} in the apical portion of the leaf in intact plants. Similar studies on excised leaf sections floated in petri dishes in darkness also gave a Q_{10} of 1.7. These findings indicate that senescence in our simpler system has a similar but probably less pronounced temperature dependence than in the invertebrate systems reported by Strehler.

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2.54 RED-LIGHT PHOTODESTRUCTION OF CHLOROPLAST ULTRASTRUCTURE

Patricia L. Walne A. H. Haber L. L. Triplett

We showed previously that a 1-megarad acute lethal dose of gamma rays produces no immediate effect on chloroplast ultrastructure in green wheat leaf tissue and that such a dose actually suppresses the slow, normal disintegration of chloroplasts that occurs in darkness.[1,2] We now report on the photodestruction of chloroplasts in wheat leaf tissue that had been given 1 megarad.

Three-centimeter apical wheat leaf tips were excised and irradiated with 1 megarad of gamma rays. Controls received no further treatment or remained in darkness for a few hours. Other leaf tips were exposed to 2 to 3 hr of red light in an apparatus in which radiation from a 750-w projection bulb was collimated, reflected, condensed, and passed through a Corning glass filter (No. 2403) that cuts off radiation with wavelength less than 600 nm. The temperature of the illuminated material was 29 to 30°C. The peak intensity was at 687 nm and ranged from about 200 to 300 $\mu w/cm^2$, depending upon position within the illuminated area. The intensities at 650 and 675 nm were 67 and 99%, respectively, of the intensity at 687 nm. Two mm-square pieces of the leaf blade were prepared for examination in the electron microscope by methods already published.[2]

We found that the sequence of events observed during photodestruction is qualitatively different from normal chloroplast disintegration in darkness or after 1 megarad of gamma rays followed by long periods in darkness. In contrast to events reported for these last two types of experiment,[2] reorientation of internal lamellae toward the periphery of the chloroplast does not occur. Accumulation of large numbers of osmophilic granules occurs only in those chloroplasts which are severely disintegrated and in grana-stroma lamellar complexes which remain after rupture of the chloroplast envelope.

The first lesions from red-light illumination appear in the stroma lamellae, which separate and swell. Subsequently, similar swelling and separation occur in the grana lamellae. Eventually, definite grana and stroma membrane configurations cannot be discerned, but continuous swollen and distorted membrane profiles traverse the entire chloroplast stroma, in line with the long axis of the plastid.

Examination of all swollen and separated lamellar profiles, grana and stroma, reveals the presence of small fibrils extending from the membranes. All effects of illumination on chloroplasts appear to be membrane centered, since the stroma regions of illuminated and dark-control chloroplasts appear to be unaffected. The 1 megarad of gamma irradiation prevents subsequent storage of absorbed light energy by the photosynthetic mechanism coupled to the enzymatic reactions of electron transport.[2] Localization of the illumination effects here reported to the internal membrane system, therefore, provides an independent line of evidence confirming other studies that indicate that chlorophyll is located in or in close association with the internal membranes. Extension of this approach to freeze-etched material might provide more-precise localization of the locus of red-light absorption.

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2.55 ABNORMAL GROWTHS IN THERMODORMANT LETTUCE SEEDS TREATED WITH GROWTH SUBSTANCES

D. E. Foard Rhonda F. Irwin

Superoptimal temperatures can prevent germination (defined as protrusion of the embryo through surrounding coats) of lettuce seeds. Such seeds are said to be thermodormant. Localized expansion without overall expansion can occur within the embryos of such seeds.[1] This localized expansion in thermodormant seeds was detected by the following: (1) crushing of single cells or small groups of cells in the radicle and the abnormally large size of cells adjacent to the crushed ones, (2) crushing of the radicle cap, (3) bulges in the vascular cylinder. We have now observed another type of expansion in thermodormant lettuce seeds, namely, abnormal outgrowths of the embryonic axis. These abnormal growths appeared in thermodormant seeds within two days after moistening with 5×10^{-5} *M* kinetin (6 of 25 examined), 3×10^{-2} *M* thiourea (2 of 22 examined), and 10^{-3} *M* indoleacetic acid (3 of 25

examined). They were not observed in thermodormant seeds moistened with 5×10^{-4} *M* gibberellic acid, nor were they present in thermodormant seeds moistened with water and exposed to red or far-red light or kept in darkness. The two thiourea-moistened seeds with abnormal outgrowths also had the first type of localized expansion listed above; the other abnormal outgrowths occurred in seeds with otherwise normal anatomy. No such abnormal growths have been observed in germinating seeds of this batch of lettuce.

Two morphological types of outgrowths may be distinguished: one having the form in section of a flattened mushroom, the other with a not so well-defined form and with a ridged or furrowed surface. These outgrowths distend the surrounding endosperm, but do not break it. They occur in localized regions of the hypocotyl about midway between the apices of the embryonic axis. Anatomically these abnormal outgrowths consist of enlarged cells of the outermost three to four rows of cortical parenchyma covered by enlarged epidermal cells; although the vascular tissue is in a parenchymoid state, at this time it does not participate in the abnormal growth. The mushroom-like type of outgrowth appears to have been formed without accompanying cell divisions. In addition to the normal developmental processes and changes that can occur in ungerminated lettuce seeds,[2] we now have demonstrated the formation of abnormal growths.

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2.56 EMBRYO EXPANSION WITHOUT ENDOSPERM RUPTURE IN LETTUCE SEED GERMINATION

A. D. Pavlista[1] A. H. Haber

Many authors have suggested that in some seeds there could theoretically be a mechanical resistance to the growth of the embryo imposed by external coats. Several authors have suggested that the endosperm acts as a mechanical restraint for germinating lettuce embryos.[2] We here report the most direct evidence for such restraint as inferred from effects of sodium dichloro-*s*-triazinetrione and sodium hypochlorite, both of which produce chlorine in aqueous solutions. These chemicals permit considerable embryo expansion without endosperm rupture in lettuce seed.

The expansion of the embryo plus the mechanical restraint of the endosperm cause the embryo to buckle. No such abnormalities are seen in similar experiments in which seeds are cut so as to remove all mechanical restraint of the endosperm. In the intact treated seeds the expanding embryo occasionally does cause endosperm rupture. When this occurs, however, the rupture is seldom near the micropylar end as in normal germination. We suggest that these two compounds interfere with a chemical endosperm weakening that normally facilitates radicle protrusion. These findings provide evidence for the separation of mechanisms initiating embryo expansion from other physiological means of endosperm weakening.

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2.57 ENVIRONMENTAL MUTAGENS – INTERNATIONAL RELATIONS

Alexander Hollaender

The environmental mutagen studies, especially synergistic effects between radiation and chemical mutagenesis, are being continued. The Environmental Mutagen Society has become extremely successful; it now has more than 300 members and is starting to play an important role in developing a program on the chemical aspects of pollution. Close cooperation has been established between the National Research Council, the Food and Drug Administration, and other government agencies in regard to this. We have been active in encouraging scientists at Argonne and Brookhaven, as well as at various universities, to take a more active interest in studying the effect of the environment. In time this endeavor should lead to the establishment of a number of regional laboratories for the study of environmental problems.

The planning work in international relations continues at a good pace. A successful and timely symposium on Fertility of the Sea was held in Sao Paulo, Brazil. The tenth symposium (1970) will be held in Santiago, Chile, and will deal with Visual Processes in Vertebrates. The eleventh will be held in La Plata, Argentina, in 1971 and will be on Protein Biosynthesis in Vertebrates. It will be conducted in cooperation with the Albert Einstein Medical School in The Bronx. The

program for this symposium is well developed. It is planned to hold the twelfth symposium in Cali, Colombia, in cooperation with Cornell University. It will deal with some basic problems in plant and animal breeding as they are related to molecular biology. The support of the local authorities in Colombia has been assured, and this promises to be one of the very important symposia. Future symposia will tend to be directed somewhat more toward applied sciences than have previous ones.

3. Genetics and Developmental Biology Section

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Mutagenesis and Cytochemistry

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Microbial Genetics and Radiation Microbiology

Roy Curtiss III
H. I. Adler

Mammalian Cytogenetics

J. G. Brewen
P. Carolyn Gooch

Somatic Cell Genetics and DNA Repair

J. D. Regan

Mammalian Cytology and Cell Genetics

E. H. Y. Chu

Mammalian Biochemical Genetics

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Drosophila Biochemical Genetics

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Drosophila Chromosomal Behavior

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Structure and Function of Multienzyme Complexes

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Cell Growth and Differentiation Unit – Tuneo Yamada
Control of Cell Differentiation

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Chromosome Ultrastructure

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Vitellogenesis

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Molecular Photobiology of DNA

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3.1 REPAIR OF DAMAGE INDUCED BY MNNG IN *HAEMOPHILUS INFLUENZAE*

R. F. Kimball Mini A. Liu[1]
Jane K. Setlow

The purpose of this program was to apply some of the techniques that have been used to analyze the molecular basis of repair of ultraviolet and x-ray damage in *Haemophilus influenzae* to damage caused by chemical mutagens. We chose *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) as the first agent to be studied because we had had previous experience with it both in *Haemophilus* and in *Paramecium aurelia* and because it is a widely used mutagen about which an appreciable amount is known in other systems.

The basic procedure has been to compare the production of damage in the wild type and four mutant strains that have defective or modified capabilities to repair uv or x-ray damage. These strains are (1) DB 112 and DB 116, independent and different mutants that are uv sensitive, unable to excise pyridine dimers, and unable to reactivate uv-irradiated phage (hcr^-); (2) DB 115, which is uv sensitive, normal in excising dimers, but slow in completing repair and also not able to reactivate phage (hcr^-); and (3) DB 117, which is uv and x-ray sensitive, able to excise dimers normally, unable to rejoin x-ray-induced breaks in DNA, and unable to support recombination in phage (rec^-).

DB 112, DB 115, and DB 116 are all slightly but significantly more sensitive than wild type to killing by MNNG. It seems probable then that the excision mechanism has a role to play in repairing potentially lethal damage induced by MNNG, though it would appear that this role might be small. DB 117 is very much more sensitive to killing by MNNG than is wild type. It is not clear just what defect is responsible for the sensitivity, but it will be shown below that it is not an inability to rejoin MNNG-induced strand breaks in DNA.

The mutation studies have been mainly on mutation from cathomycin sensitivity to cathomycin resistance. We have been unable to demonstrate any differences between the strains in the rate of MNNG-induced mutation. If any difference exists at all, it is a slight *decrease* in the sensitivity of DB 112. There is certainly no increase in sensitivity. It is particularly notable that DB 117, which is very sensitive to killing, is not sensitive to mutation induction. This agrees with findings on *Escherichia coli* for similar rec^- mutants and shows that the system involved in excess killing in this stock is not involved in any major way in the mutation process.

A study of DNA strand breaks, using the method of McGrath and Williams to lyse bacteria directly on sucrose density gradients, has confirmed the work of others with *E. coli* that MNNG treatment produces such breaks and that they disappear when the bacteria are incubated after treatment and before lysis. The breaks are produced and disappear about equally in all strains, even in DB 117, which is unable to repair x-ray-induced breaks. Thus the repair involved is neither excision repair nor rejoining of breaks by a mechanism identical to that by which x-ray breaks are rejoined. It is not certain whether the damage is actually present as real breaks in the bacteria or is produced from some other form of damage to DNA during lysis and centrifugation. In any case, this type of damage cannot be responsible for the excess killing in DB 117. At the moment, it is possible that this damage is responsible for mutation since neither mutations nor breaks are appreciably affected by the defects in the uv-sensitive stocks. Of course, this negative evidence only raises a possibility and does not establish a true correspondence.

Treatment of DNA outside the cell with high doses of MNNG produced at most a relatively small amount of inactivation of transforming ability and no detectable mutation, whether the DNA was used to transform the wild type or the excisionless strains.

Reference

[1] Student trainee.

3.2 COMBINED MICROPHOTOMETRIC AND AUTORADIOGRAPHIC PROCEDURES FOR STUDYING ALTERATIONS OF THE CELL CYCLE IN MAMMALIAN CELLS

R. F. Kimball Stella W. Perdue

In many instances during the changes that occur in normal development, in neoplastic transformation, and in cultures of cells, it would be desirable to follow in detail the changes in the cell cycle as the growth rate of the cells varies. The usual autoradiographic procedures for studying the cell cycle are difficult to apply to very prolonged cycles or to cycles during which major changes in growth rate are occurring. Moreover, the autoradiographic studies make it possible to define the major periods of the chromosome replication cycle (G_1, S, G_2, and M) but do not allow a more fine-grained study such as would be needed when the great majority of the cells are in one stage, G_1, for example.

We have developed a procedure based on a combination of microspectrophotometry, microinterferometry,

and autoradiography that makes a much more detailed analysis possible. Cells are pulse labeled with tritiated thymidine, fixed in alcohol-acetone, and stained with a protein stain, dinitrofluorobenzene (DNFB). This stain has proved very stable to all further treatment and very suitable for microspectrophotometric determinations of total cell protein. Autoradiographs are prepared, and the locations of unlabeled and labeled cells are marked on photographs of selected fields for identification after removal of the silver grains. The cells are then stained for DNA by the Feulgen procedure, which removes the grains and prepares the cells for photometric measurement. The total extinction (absorbancy integrated over the whole cell) of individual cells is measured at two wavelengths, one heavily absorbed by DNFB and one by Feulgen, using a Zeiss UMSP scanning microspectrophotometer. Samples of unlabeled, labeled, and mitotic cells are included so that the distribution of total protein content and DNA content in these three classes of cells can be determined. The unlabeled nonmitotic cells include both G_1 and G_2, but these can be separated by their DNA content. The mitotic cells provide a measure of the DNA and protein content at the end of the cell cycle, and by halving, at the beginning as well. The continuous increase in protein content during the cycle allows a distinction between early and late G_1 cells and makes it possible to relate the rate of DNA synthesis to the rate of cell growth.

We have also found it advantageous to measure the dry mass of samples of fixed, unstained cells by scanning microinterferometry. This provides an independent check on the adequacy of the protein stain as a measure of cell size. There has been very good agreement between the two methods when populations with different cell sizes have been compared, and the amount of stain bound per unit mass has been shown not to differ appreciably under widely varying growth conditions.

We acknowledge gratefully Dr. E. H. Y. Chu's help in supplying Chinese hamster cells to use in establishing this method.

3.3 THE EFFECT ON THE CELL CYCLE OF THE SHIFT FROM THE EXPONENTIAL TO THE STATIONARY PHASE OF CULTURED CHINESE HAMSTER CELLS

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Stella W. Perdue

A major factor of normal development is the controlled inhibition of cell multiplication, and it is the breakdown of this inhibition that is characteristic of neoplasms. Rapidly growing tissues or cultures consist of a mixture of cells in various stages of the cell cycle. How is such a population converted to an inhibited population, growing slowly if at all? Is there a block at a specific stage of the cell cycle with consequent piling up at this stage, or are cells in all stages nearly equally inhibited, leaving an inhibited population with a mixture of stages? Answers to such questions should give us a better idea where to look for the controls for normal inhibition. Past studies by many workers have suggested an accumulation of cells in the G_1 stage of the cycle, but it was far from clear whether this involved a fairly specific block or whether there was still a complete set of cycle stages, but with an increase of G_1 relative to the other stages.

We decided to investigate this matter first in a growth inhibition situation common to cultured mammalian cells and microorganisms, namely, the inhibition of growth that occurs when a culture is allowed to grow without renewal of the medium. The cell cycle was studied by the combined microphotometric and autoradiographic method described in the preceding report. Indeed this study was undertaken, in part, to test the utility of this method.

Exponentially growing cultures of Chinese hamster lung cells were compared with five- and six-day-old cultures. The mitotic index of the five-day cultures was only a few hundredths of a percent, compared with 2% for the exponential phase, and that of the six-day culture was still lower, showing that both cultures were far along into stationary phase.

The combined microphotometric and autoradiographic studies allow the following conclusions to be drawn:

1. As was already known for both microorganisms and cultured mammalian cells, growth in cell mass decreases before cell multiplication rate; consequently the size of the cell at division and the average cell size in the population decrease.

2. The onset of DNA synthesis is delayed relative to the length of the whole cell cycle; consequently G_1 comes to occupy an increasing fraction of the cycle.

3. At first the delay is such that the cell size at the onset of S remains very nearly at its exponential-phase value, despite the distinctly smaller size at mitosis.

4. As cell size decreases still further as a result of continued division with very little growth, the size at the onset of DNA synthesis also decreases. There is no evidence in our results for a lower limit of size

below which DNA synthesis cannot be initiated, though there must be some lower limit to the decrease in cell size.

5. The distribution of cell sizes, when expressed as a percent of the mean cell size, remains nearly constant even though the mean cell size decreases to about half its exponential-phase value.
6. Cells in S and mitosis, though infrequent, are still found at six days.
7. Autoradiographic studies suggest that the cells passed through the cycle during the interval between five and six days at the rate of approximately 0.4 of a cycle per day.

Thus the population retains a complete series of cell cycle stages as judged by cell size and by the occurrence of S and mitosis, though with a great increase of G_1 relative to the rest of the cycle. There is no evidence of a true block in any stage, only a shift in the rate of completion of preparation for DNA synthesis vs preparation for cell division. Presumably such a population can readily return to the state characteristic of exponential growth by speeding up the various component processes in the reverse order in which they were slowed down. We would expect this to be a model for the way in which a cell population would behave anytime its growth is inhibited by a limitation of substrates.

3.4 CONJUGATION WITH DNA-ts STRAINS OF *ESCHERICHIA COLI* K-12

D. R. Stallions Roy Curtiss III
Gary Van Denbos[1]

In 1966, Bonhoeffer[2] reported that F^- strains of *Escherichia coli* K-12 possessing a DNA-ts mutation were unable, during bacterial conjugation, to act as recipients of donor DNA when matings were conducted at the restrictive temperature for DNA synthesis. The presence of a DNA-ts mutation in the Hfr donor parent had no effect on chromosome transfer for matings at any temperature. Bonhoeffer therefore concluded that DNA synthesis was required in the F^- recipient for chromosome transfer to occur – a conclusion which is contrary to all other published data.

In order to study further the role of DNA synthesis during conjugation, we obtained from Bonhoeffer a derivative of his F^- DNA-ts mutant. We isolated appropriate genetically marked derivatives from this strain and also prepared a series of Hfr, F′, F^+, and F^- strains possessing the DNA-ts mutation by conjugation. The phenotypes of recombinants obtained in these matings with respect to temperature sensitivity varied considerably. This suggested that the original DNA-ts strain might possess multiple mutations affecting thermosensitivity. Therefore we chose strains that grew well at 35°C but not at temperatures above 40°C and that stopped synthesizing DNA within 1 to 2 min after shifting to 43°C. The strains chosen were also capable of being transduced to the temperature-resistant state by the transducing phage P1. This ability indicates a probable single mutation or very closely linked mutations controlling temperature sensitivity.

In most matings involving a great variety of Hfr donors having different origins and directions of chromosome transfer and DNA-ts recipients, the efficiency of genetic transfer at 43°C was equal to or greater than that at 35°C, a finding in direct contradiction to the results reported by Bonhoeffer. An exception to this general result was observed in experiments in which Hfr H (the only donor strain used by Bonhoeffer) was mated with a DNA-ts F^- strain. In this case the frequency of recombinant formation at 43°C was 0.1 to 0.01% of that at 35°C. An investigation of the kinetics of chromosome transfer and recombinant production in matings between Hfr H and DNA-ts F^- strains at 43°C has shown that transfer of Hfr H DNA is unaffected by the restrictive temperature but that the expression and/or integration of transferred genes is reduced. The recombinant yield for any given donor marker for matings conducted at 43°C is dependent on the time of marker transfer, with decreases in recombinant yield first detectable 20 min after commencement of marker transfer; maximum reductions in recombinant yield are attained about 40 min after commencement of marker transfer.

Experiments with all DNA-ts Hfr donors revealed no effect on chromosome transfer and recombinant formation for matings with DNA-ts$^+$ F^- strains conducted at 43°C, a result in agreement with those reported by Bonhoeffer. In matings conducted at 43°C between an Hfr H DNA-ts strain and a DNA-ts F^-, reduced recombinant yields were obtained that were identical to those in matings between Hfr H (DNA-ts$^+$) and a DNA-ts F^-. In all other matings between Hfr DNA-ts donors and DNA-ts F^- recipients, the frequency of chromosome transfer and recombinant production at 43°C was equal to or greater than that at 35°C. These results indicate that the results with DNA-ts$^+$ Hfr donors were not due to the transfer of the DNA-ts$^+$ gene or to a product of the DNA-ts$^+$ gene.

We feel that our results with the original DNA-ts strain supplied by Bonhoeffer and with other DNA-ts

Hfr and F^- strains indicate that DNA synthesis in the recipient is not necessary for chromosome transfer and that the original results reported by Bonhoeffer for matings with Hfr H and the DNA-ts F^- were due to an effect on recombinant formation and not on chromosome transfer.

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

[2] F. Bonhoeffer, *Z. Vererbungslehre* **98**, 141–49 (1966).

3.5 CONJUGATION WITH MINICELLS ISOLATED FROM A DNA-ts MINICELL-PRODUCING F^- STRAIN OF *E. COLI*

Roy Curtiss III R. L. Siegel[1]

The DNA-deficient minicells derived from the F^- minicell-producing strain P678-54[2] accept, during conjugation, single-stranded F DNA transferred by radioactively labeled F^+ donors.[3] The minicells are capable of converting this single-stranded DNA to double-stranded DNA, and they are known to contain the Kornberg DNA polymerase and the polynucleotide-joining enzyme.[3] Hfr donors also transfer single-stranded DNA to minicells during conjugation, but in this case the minicells are unable to convert this single-stranded DNA to double-stranded DNA.[3] We were interested in knowing whether minicells derived from a DNA-ts minicell-producing F^- strain could accept isotopically labeled F and Hfr DNA at the restrictive temperature for DNA synthesis and, if so, whether they were capable of converting single-stranded F DNA to double-stranded F DNA at this temperature. A DNA-ts derivative of P678-54 was prepared containing the Bonhoeffer DNA-ts mutation.[4] The minicell-producing parent cells of this strain were shown to accept genetic material during conjugation at both 43 and 35°C. DNA synthesis in this strain ceases within 1 to 2 min after shifting from 35 to 43°C, whereas protein and RNA synthesis continue at near-normal rates for about 30 min. Transfer of isotopically labeled F DNA occurred at both 35 and 43°C, corroborating our other evidence that DNA synthesis in the recipient is not essential for genetic transfer. Following such matings at 35 and 43°C, minicells were purified and then incubated with a second label at either 35 or 43°C. Incorporation of this second label into DNA occurred at both temperatures. This suggests that the DNA-ts mutation does not result in any thermosensitive products involved in the in vivo conversion of single-stranded F DNA to double-stranded F DNA.

As a test for an F-controlled DNA synthesis associated with chromosome transfer by Hfr donors, we mated ^{3}H-thymidine-labeled DNA-ts Hfr cells with DNA-ts minicells at 43°C in the presence of ^{14}C-thymidine. The amount of ^{3}H-thymidine-labeled Hfr DNA transferred to minicells was about equal to the amount of ^{14}C-thymidine-labeled DNA synthesized in the Hfr during mating. These results indicate that DNA transfer during conjugation is accompanied by DNA synthesis in the Hfr parent and that this DNA synthesis and synthesis during vegetative chromosome transfer are under separate control. In all probability, therefore, genetic transfer is dependent on DNA synthesis in the donor parent and is independent of DNA synthesis in the recipient parent.

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[1] Student trainee.

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[4] F. Bonhoeffer, *Z. Vererbungslehre* **98**, 141–49 (1966).

3.6 HYBRIDIZATION STUDIES ON DNA TRANSFERRED TO MINICELLS: A POSSIBLE METHOD FOR THE ISOLATION OF SPECIFIC REGIONS OF THE BACTERIAL CHROMOSOME

J. A. Fralick[1] W. D. Fisher

In recent studies concerned with the molecular aspects of chromosomal transfer during bacterial conjugation in *Escherichia coli* K-12, Cohen *et al.*[2] and Ohki and Tomizawa[3] have presented data that are quite suggestive that donor strains transfer a single "old" strand of their DNA to recipient cells. Furthermore, Ohki and Tomizawa[3] and Rupp and Ihler,[4] using the lambda prophage system, have reported that a donor DNA strand which had been labeled prior to mating and contained a 5′ terminus at the origin was asymmetrically transferred during bacterial conjugation. These studies, therefore, predict that the single-stranded DNA transferred during conjugation by two Hfr donors which transfer their chromosomes in opposite directions would be complementary only at the specific regions which genetically overlap. Unfortunately it is no simple matter to isolate the recipients from the donors

in a typical *E. coli* mating mixture, let alone to isolate the newly transferred DNA strand(s). Yet we have a system which enables us to do just that – to isolate DNA transferred during bacterial conjugation.[2] This system is composed of small anucleate structures called "minicells," which are readily separable from normal-sized *E. coli* by either differential or rate sedimentation centrifugation.[5] Therefore we investigated the question as to the uniqueness of the DNA transferred to minicells by Hfr donor strains. Hybridization studies on DNA strands isolated from minicells mated with Hfr strains which transfer the *proC* region of the chromosomes near their origin but in opposing directions suggest that the transferred DNA represents a unique strand. These results support the findings of Ohki and Tomizawa[3] and of Rupp and Ihler[4] on asymmetric chromosome transfer during bacterial conjugation and of Cohen *et al.*[2] with respect to the recovery of predominantly single-stranded DNA from minicells which had been mated with Hfr donor strains. Our findings also suggest a possible method for the isolation and purification of specific regions of the bacterial chromosome. In other words, the minicell system enables us to isolate specific regions of specific strands of the bacterial chromosome. Therefore, by using appropriate Hfr or F′ strains, one could isolate, from minicells, DNA strands which would be complementary only at a specific region, and hence by hybridization and digestion of single-stranded DNA segments by specific nucleases, it should be possible to isolate specific regions of the bacterial chromosome.

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3.7 NUCLEAR MORPHOLOGY AND BEHAVIOR IN LIVING *ESCHERICHIA COLI* CELLS

H. I. Adler Alice A. Hardigree

We have reported previously some attempts to follow, by means of time-lapse cinematography, the growth, division, and segregation of nuclear masses in living cells of the bacterium *Escherichia coli.* In spite of the fact that the nuclear masses are no more than a few tenths of a micron in diameter, our preliminary results were good enough to allow us to discern at least two patterns of nuclear division and segregation. Encouraged by these results, we have spent the last year in trying to improve our biological and microscopic techniques to further resolve details of nuclear morphology and behavior. Recent progress has been made by modifying the growth medium for the cells and by making use of anoptral phase contrast optics and sensitive color films. With these improved techniques, certain discrepancies between the concept of the bacterial nucleus derived from genetic and molecular biology approaches and that obtained by direct observation of living cells become very impressive. For example, direct observation suggests that the entire nuclear mass undergoes a central constriction that results in the separation of the two daughter nuclei. It is difficult to reconcile this with the notion that DNA double helices are separated as a result of growth between points of attachment between DNA and the cell membrane. This latter concept would suggest a gradual unraveling of the nuclear body into two nuclear bodies. Both the time-lapse films and thin-section electron micrographs do not support such a concept. We are also currently investigating the effects of changes in tonicity of the growth medium on the morphology of the nucleus.

3.8 GENETICS OF THE MINICELL PHENOTYPE IN *E. COLI* K-12

K. J. Roozen[1] Roy Curtiss III

Experiments were undertaken to determine the chromosomal location of the mutation conferring the ability to produce minicells in *Escherichia coli* K-12. Although preliminary work suggested that the minicell mutation (*min*$^-$) was near the *lon* locus in the chromosomal region between the *proC* and *purE* loci,[2] the transfer and integration of this region by conjugation between an F$^+$ *min*$^-$ strain and several F$^-$ *min*$^+$ strains did not yield minicell-producing recombinants. Minicell-producing recombinants were formed when the *proC-purE* region of an F$^+$ *min*$^-$ strain was transferred into the F$^-$ strain from which the *min*$^-$ mutant originated. Subsequent experiments yielded minicell-producing recombinants if the region between *proC* and *pyrD* was transferred from an F$^+$ *min*$^-$ strain to several F$^-$ *min*$^+$ strains. These results suggested that more than one altered locus was responsible for the minicell phenotype, one located in the *proC-purE* region and the other

in the *pdx-pyrD* region. This hypothesis receives further support from experiments demonstrating that if either region suspected of harboring a *min* locus is removed singly from a *min*$^-$ strain, the ability to produce minicells is lost.

References

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

[2] F. Bonhoeffer, *Z. Vererbungslehre* **98**, 141–49 (1966).

3.9 STUDIES ON A BACTERIAL HYBRID SYSTEM FOR ISOLATING SPECIFIC REGIONS OF THE *E. COLI* K-12 CHROMOSOME

K. J. Roozen[1] Roy Curtiss III D. R. Stallions
Annie S. Angel

Bacterial hybrids useful for isolating specific regions of the *Escherichia coli* K-12 chromosome can be formed by the conjugal transfer of F′ factors from *E. coli* to mutant recipient strains of *Proteus mirabilis.* After extraction from the hybrid, the F′ DNA can be separated from the *P. mirabilis* DNA by a variety of physical techniques by making use of the facts that *E. coli* DNA has a 50% G-C content and *P. mirabilis* a 39% G-C content and that the F′ DNA can be extracted as closed circular DNA.

We have investigated numerous mating techniques, culture media, selective media, and mutagenesis procedures in order to facilitate the preparation of the hybrid strains. To date, 12 genetically different *E. coli* F′ factors are available in 33 different *E. coli* F′–*P. mirabilis* strains. About 60% of the *E. coli* K-12 chromosome is represented collectively in the hybrid strains prepared so far. In order to verify the existence of a hybrid strain, we have relied on analytical CsCl ultracentrifugation and the ability of a hybrid to transfer its F′ to a suitably marked *E. coli* recipient. It is of interest to note that the transfer of an F′ *lac*$^+$ episome from a hybrid strain to an *E. coli lac*$^-$ recipient occurs with a higher frequency in crosses with a restriction- and modification-deficient (r^-m^-) recipient than with an r^+m^+ recipient. This suggests, as might be expected, that *E. coli* F′ DNA harbored in *P. mirabilis* has a modification pattern different from the pattern in *E. coli* K-12. When either r^-m^- or r^+m^+ *E. coli* F′ *lac*$^+$ donors were used in crosses with *lac*$^-$ *P. mirabilis* recipients, there were no differences in the frequency of F′ *lac*$^+$ transfer. The investigation of the role of restriction and modification phenomena in the formation of *E. coli–P. mirabilis* hybrids will be continued with an attempt to isolate r^-m^- mutations in *P. mirabilis.* It is hoped that mutations of this type will enable us to prepare hybrid bacterial strains more easily.

Although a number of possible studies utilizing this system for isolating specific regions of the *E. coli* chromosome have become evident, we have only become involved in two studies while continuing to develop the system. Since previous unpublished data suggest that the rRNA genes are located in the *ilv* to *thi* region of the *E. coli* chromosome, we are using a variety of *E. coli* episomes containing different segments of the *ilv-thi* region of the K-12 chromosome to determine a more exact location of rRNA genes. This will be done by measuring the amount of rRNA, isolated by sucrose density gradient sedimentation, that will hybridize with different episomal DNA preparations having known genetic contents. This study is being done in collaboration with Dr. S. Kaplan of the University of Illinois. The mapping of tRNA genes using similar techniques has also been considered.

A second investigation, using hybrid strains to obtain DNA from a variety of K-12 chromosomal regions, has been initiated in collaboration with Dr. Robert Bird. This study involves a comparison between DNA's extracted from rapidly growing and from stationary-phase cultures of *E. coli* K-12 for hybridization to F′ DNA in order to compare the number of copies of specific regions of the chromosome for cells grown under these two conditions. After completing these studies we intend to examine *E. coli* cultures in which amino acid starvation procedures have been utilized to "synchronize" the replication of chromosomal DNA. These studies will provide information on the origin (or origins) and direction of chromosome replication as well as on the number of growing forks present in a replicating chromosome for cells growing under various conditions.

Reference

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

3.10 CYTOGENETIC STUDIES OF THE VILLAGERS OF ANAKTUVUK PASS, ALASKA

J. G. Brewen

Long-term monitoring of the Arctic Eskimos has established that these people have an excessive body burden of radionuclides, with the average ^{137}Cs burden of adults being 50 to 60 times higher than the average

for adults not directly involved in the Arctic food chain.

Short-term peripheral lymphocyte cultures were made from whole-blood samples taken from 21 male villagers at two different times (February and August) during calendar year 1969. The sampling times coincided with the yearly low and high body burdens respectively. Colchicine-arrested metaphase chromosome preparations from these cultures were analyzed for evidence of a cytogenetic effect of the high body burdens of radionuclides. Concomitant with the August sampling at Anaktuvuk Pass, 11 samples were taken from villagers at Kotzebue to serve as controls, since their body burdens of ^{137}Cs are considerably lower due to the small amount of caribou meat in their daily diets.

Tables 3.10.1 and 3.10.2 summarize the cytogenetic findings in all of the samples as well as the body burdens, where available, at the time of the sampling.

In assessing the significance of the data, several factors must be weighed. First, in a study of this kind, chromatid-type aberrations are not very meaningful due to the high (2–4%) frequency of spontaneously occurring ones in cultures with no history of radiation exposure. Second, the class of aberrations classified as chromosome deletions cannot be weighed heavily because many of them are (doubtless morphologically

Table 3.10.2. Kotzebue Data

Individual	No. Cells Scored	No. Rings and Dicentrics	No. Deletions	No. Chromatid Breaks
F. Miller	300	0	0	3
L. Williams	300	0	4	3
H. Monroe	300	0	1	6
J. Stein	300	0	0	8
L. Norton	300	0	1	2
I. Mendenhall	300	0	0	2
Y. Mendenhall	300	0	2	3
K. Kinneaveak	300	0	6	4
N. Koenig[a]	300	1	3	7
C. Sampson	300	0	1	3
A. Porter	300	0	2	4
Total	3300	1	20	45

[a]This individual is the x-ray technician at the U.S.P.H.S. Hospital at Kotzebue.

Table 3.10.1. Anaktuvuk Pass Data

Name	Spring Burden (nc)	No. Cells Scored	No. Rings and Dicentrics	No. Deletions	No. Chromatid Breaks	Fall Burden (nc)	No. Cells Scored	No. Rings and Dicentrics	No. Deletions	No. Chromatid Breaks
D. Mekiana	410	300	4	2	3	860	300	0	1	4
J. Mekiana	400					1100	300	0	2	6
P. Mekiana	80	300	0	0	14	*a*				
K. Mekiana	90	300	0	0	8	600	300	0	2	10
A. Paneak	60	300	0	1	8	*a*				
S. Paneak	250	350	1	6	3	1350	300	1	2	2
R'bt. Paneak	270	300	0	0	8		300	0	0	5
R'svlt. Paneak	220	300	1	0	2	810	300	0	0	6
R'mnd. Paneak	*b*					970	300	1	0	6
S. Rulland	190	300	3	0	5	700	300	0	1	14
T. Rulland	180	300	0	0	8	720	300	0	0	8
J. Rulland	270	300	0	2	5	1300	400	0	2	12
John Rulland	*b*					780	250	1	0	5
L. Rulland	210	300	0	2	2	1150	200	0	0	2
R. Ahgook	310	300	1	4	2		300	2	1	6
A. Morry	270	300	2	2	6	950	300	0	1	6
J. Morry	430	300	0	3	5	1200	200	0	0	1
B. Morry	*b*					840	300	0	0	5
C. Hugo	240	300	1	4	8	760	350	3	0	8
J. Hugo	*b*					910	300	1	0	12
E. Kakiana	300	300	0	4	5	*b*				
Total		4,850	13	30	92		5,300	9	12	118

[a]Teenage boy.

[b]Not in village at time of sampling.

identical) nonunion isochromatid deletions, a class of aberrations with a reasonably high (0.5–1.0%) spontaneous frequency. Third, control dicentric and ring frequencies can be misleading because those seen by us in laboratory control personnel are generally "overscored" and of the clone type, that is, an identical aberration appearing in more than one cell from the same individual.

The dicentric and ring frequency (0.22%) in the Anaktuvuk Pass villagers is significantly higher than that seen in persons with no history of exposure to excessive ionizing radiation. In fact, the yield is quite comparable with that reported by Buckton *et al.*[1] for radiation workers who received doses of 1 to 10 rads, with a mean of 3.8, over a period of ten years or more. Of the 22 rings and dicentrics observed, 9 had a single fragment associated with them and thus were probably produced in the circulating blood. The other 13 aberrations either had no fragment or had two identical fragments and were in all probability "old" aberrations induced in the bone marrow.

Since there would be no expected significant two-track contribution to aberration production due to the chronic nature of the radiation, biological dose estimates should be made on the basis of the coefficient of the one-track term of the quadratic expression $Y = a + bD + cD^2$. Using Bender's and Evans' estimates of b (see Evans[2]), a dose of 0.25 to 2.5 r per year is arrived at for the nine "new" aberrations.

Actual physical dose estimates are currently being calculated by the Mammalian Ecology Division, Battelle Memorial Institute, Hanford, Washington.

The one dicentric chromosome observed in the Kotzebue controls was seen in a blood sample from the x-ray technician at the U.S.P.H.S. Hospital and, therefore, should be viewed with skepticism.

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3.11 CYTOGENETIC EFFECTS OF CYCLOHEXYLAMINE AND N-HYDROXYLCYCLOHEXYLAMINE

J. G. Brewen F. G. Pearson Katherine P. Jones
H. E. Luippold

The artificial sweetening agents sodium cyclamate and calcium cyclamate are known to be metabolized to form cyclohexylamine and N-hydroxylcyclohexylamine in a significant portion of the human population. It has been reported that the treatment of both a human and a marsupial cell line with cyclohexylamine results in observed chromosomal damage in the treated cells. Furthermore, injection of as little as 5 mg of cyclohexylamine per kilogram of body weight into rats is purported to induce a significant amount of chromosomal damage in the bone marrow and spermatogonial cells of the recipient animals.

We have investigated the supposed cytogenetic effects of cyclohexylamine on human peripheral lymphocytes in culture as well as via a host-mediated assay system we developed. The animal used in the host-mediated assay tests was the Chinese hamster. In addition to the human lymphocytes, bone marrow preparations of the host animal were analyzed for chromosome damage. The cytogenetic effects of N-hydroxylcyclohexylamine on human lymphocytes in tissue culture were also studied.

In all, six experiments were done testing the possible cytomutagenicity of cyclohexylamine. The in vitro doses ranged from 20 to 500 μg per milliliter of culture medium, and the cells were exposed to the chemical at various stages of the cell cycle. A total of 19 experimental treatments were given in vitro, and 4600 metaphase figures were analyzed. As in the case of cyclohexylamine, no significant cytogenetic effect was found. The doses of N-hydroxylcyclohexylamine ranged from 2 to 250 μg/ml, and all stages of the cell cycle were treated.

The host-mediated assay tests are not complete at this time, but the existing data show no cytogenetic effect of cyclohexylamine on either the human lymphocytes or host animal's bone marrow at doses up to 450 mg per kilogram of body weight.

These results are in conflict with those published by other workers, but in light of the fact that the previous reports showing a positive effect did not include the actual data, it is difficult to explain the discrepancy.

3.12 FURTHER STUDIES OF XERODERMA PIGMENTOSUM INCLUDING THE DeSANCTIS-CACCHIONE SYNDROME; HUMAN GENETIC DISEASES RESULTING IN ULTRAVIOLET-INDUCED SKIN CANCER

James D. Regan W. H. Lee
R. B. Setlow James German[1]
W. L. Carrier Edmund Klein[2]

We have previously shown the lack of functional uv endonuclease to be the fundamental defect in xeroderma pigmentosum (XP) (see Sect. 6.41, this report).

We have extended these studies to include three more affected individuals of a different kindred from that of our first patient. The results from this study confirm our previous work. XP cells do not excise uv-induced pyrimidine dimers from their DNA because they fail to make the initial single-strand breaks next to the dimers. The failure to perform this step is due to the lack of the endonuclease. Results with the three additional patients were identical within the error of the determinations. We have also studied cells from two patients with the DeSanctis-Cacchione syndrome. This condition is considered by some workers to be a variant of XP, since these individuals are subject to uv-induced skin carcinomas. However, this condition is obviously more complex, because DeSanctis-Cacchione patients also exhibit severe neurological problems involving mental retardation and motor difficulties. Our studies with DeSanctis-Cacchione cells show that they do not excise dimers and do not appear to have a functional uv endonuclease, a result which may, in view of our data from uncomplicated XP cells, suggest a reason for the skin carcinoma. The basis for the neurological difficulties is unknown at present. It is conceivable that the DeSanctis-Cacchione syndrome may be due to a deletion involving not only the locus specifying the uv endonuclease but also loci involved with normal neurological development.

We have also investigated repair phenomena in Bloom's syndrome, another disease involving uv sensitivity but without the development of skin carcinoma. Fibroblasts from an individual with this disease do show dimer excision and strand breakage. The molecular basis of this disease is apparently not the lack of a uv endonuclease but possibly a defect at some later repair step.

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3.13 RADIATION AND CHEMICAL MUTAGENESIS IN MAMMALIAN CELLS IN CULTURE

E. H. Y. Chu Patricia A. Brimer
E. G. Bailiff

With the availability of genetic markers[1] and the demonstration of chemical mutagenesis[2] in tissue culture mammalian cells, it becomes feasible not only to explore the basic mechanisms of mutagenesis[3] but also to apply the cell culture system for test of mutagenicity of chemical and physical agents. In 1969 we have extended some of the earlier studies and initiated several other lines of approaches. Some of the new findings are summarized in the following sections.

Our previous work[1] shows that the hamster cells were sensitive to 8-azaguanine at concentrations greater than 1 μg/ml. Resistance to the analog arose from a loss or reduction in the activity of hypoxanthine guanine phosphoribosyl transferase. In Lesch-Nyhan syndrome in man, the control of the same enzyme activity is x linked.

In hamster cells both spontaneous and chemically induced forward and reverse mutation occurred at this locus. The rate of spontaneous forward mutation to azaguanine resistance was 1.5×10^{-8} per cell per generation. In the experiments to be discussed below, the forward mutagenic change at this locus in the hamster cells was determined. Parallel studies have been initiated to use normal skin fibroblasts from human male and female donors.

Induction of Forward Mutations with X Rays. – Hamster cells were exposed to x rays from 0 to 1200 r. The x-ray survival of these cells fits approximately a multitarget relation. The result is in close agreement with those of other investigators for the same cell line.

Within the x-ray dose range tested, the frequency of induced forward mutations (expressed as the function of survivors) increases significantly and exponentially with dose. The data indicate that forward mutation to a presumably sex-linked recessive is a single event at doses below 400 r. At higher doses, however, the mutant frequencies appear to increase in proportion greater than the first power of dose and then begin to decrease due possibly to a saturation effect.

Mutagenicity of Metabolic Derivatives of Cyclamates. – The possible mutagenic effect of two metabolic products of cyclamates, cyclohexylamine (CHA) and N-hydroxylcyclohexylamine (N-OH-CHA), were tested in the hamster cells in culture. CHA at a concentration as high as 10^{-2} *M* reduced cell survival to approximately 50%, but there was no increase of mutation frequency over the control even with further cell killing. N-OH-CHA was about 10 times more toxic than CHA, and the killing curve was extremely steep in the concentration range from 6×10^{-4} *M* to 1×10^{-3} *M*. When the cell survival was reduced to less than 20% there was a dramatic increase in the frequency of mutations over the control. No significant increase in mutation frequency was found at higher levels of cell survival. Under the experimental conditions it can be concluded that CHA was not mutagenic, whereas N-OH-CHA was weakly mutagenic. Tests with other loci

in the same cells as well as with different cell types are in progress to confirm and extend these observations.

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3.14 FORWARD AND REVERSE MUTATIONS IN THE RESISTANCE OF CHINESE HAMSTER CELLS TO 5-BROMODEOXYURIDINE

E. H. Y. Chu Ti Ho

Incorporation of 5-bromodeoxyuridine (BUdR) into Chinese hamster cells ($2n = 22$) in culture causes a reduction in cell survival that is proportional to the amount of incorporation and posttreatment with visible light. A subline resistant to 10^{-3} *M* BUdR was obtained after mass cell cultures were exposed to increasingly higher concentrations of the analog. The modal chromosome number of the subline was 18, and the karyotype differed considerably from the parental cells. Autoradiographic evidence showed that the resistant cells were incapable of incorporating ^{3}H-thymidine. BUdR resistance was shown to be a recessive character in somatic cell hybrids.

Fourteen different clones were isolated. Each retained the resistant character and the same chromosome number after prolonged maintenance in BUdR-free medium. Fluctuation tests of a clone showed that reversion to BUdR sensitivity occurred randomly, suggesting that this change was probably due to spontaneous back mutation and that the locus was not deleted in this clone despite karyotypic deviations from the normal. The spontaneous back-mutation rate was estimated as 4.8×10^{-8} per cell per generation.

Reversion studies of all the resistant clones, however, revealed differences in revertibility to BUdR sensitivity. Some clones reverted readily, either spontaneously or after treatment with alkylating agents (e.g., EMS and MMS); others were totally nonrevertible. It is possible that during original mass selection several types of forward mutational events had occurred, all leading to BUdR resistance. These events might include single nucleic acid base substitutions and gross deletions, both of which could be the result of the action of BUdR. It is further apparent that hemizygosity as a consequence of loss of a chromosome or a chromosome region may facilitate the expression of new forward mutations.

3.15 AMINO ACID SEQUENCE IN THE BETA CHAIN OF MOUSE HEMOGLOBIN

R. A. Popp

The sequence of amino acids in a portion of the beta chain of C57BL hemoglobin has been reported.[1] The remainder of the tryptic peptides has been studied. The proposed sequences[1] of βT-4 and βT-5 were found to be correct; however, the proposed sequences for βT-2, βT-10, and βT-12 were incorrect. The correct sequences are as follows: βT-2 is Ser Ala Val Ala Gly Leu Try Gly Lys, βT-10 is Gly Thr Phe Ala Leu Ser Glu Leu His Cys Asp Lys, and βT-12 is Leu Leu Gly Asn Met Ile Val Ile Val Leu Gly His His Leu Gly Lys. The β chain of C57BL hemoglobin has 146 residues and differs at 25 residues from the β chain of human hemoglobin A.

Reference

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3.16 TRYPTIC PEPTIDES OF MOUSE ERYTHROCYTE STROMA

R. A. Popp Diana M. Popp

Although it has not been possible to purify any one of the proteins that make up the cell membrane, we thought that considerable information might be learned about some properties of these proteins from the peptides that result from the tryptic digestion of stroma. Much of the protein in erythrocyte stroma is refractory to digestion by trypsin. Aminoethylation of the cysteine residues improves the quality of digestion, but an insoluble core, which represents about one-third of the total stroma, still remains.

Fingerprints of the soluble tryptic peptides of stroma from different strains of mice show a marked similarity in most peptides, but some unique peptides were observed. The premise is that the variant peptides are segments of polypeptide chains controlled by alleles at any one of several loci, for example, H-2, H-14, Es-3, which specify proteins found in the membrane. Separations of the peptides by gel filtration over Sephadex

G-25, column chromatography, paper chromatography, and high-voltage paper electrophoresis reveal that there are more than 200 peptides in the tryptic digests of mouse stroma. To date no specific peptide variation can be associated with the H-2 serotype of mice.

3.17 STUDIES ON MURINE ERYTHROCYTE STROMA

R. A. Popp Mary W. Francis

Membranes must be solubilized in order to separate individual proteins from a complex mixture of proteins like those found in cell membranes. Moreover, the procedure for solubilization should be mild enough so that peptide bonds are not affected. Stroma can be solubilized in 8 *M* urea, β-mercaptoethanol, and Tris-HCl, but the product is very insoluble after urea is removed by dialysis. Formic acid, 0.7 *M*, will also dissolve stroma, and some glycoproteins remain in solution after formic acid is removed. A soluble preparation of membranes can be obtained by using a biphasic water-butanol extraction procedure.[1] The protein in the water phase can be concentrated to 10 mg/ml by pressure dialysis before a flocculation appears. Use of lyophilization to concentrate the protein yields a highly insoluble preparation. Salts, even 0.85% NaCl, reduce the solubility of the protein. Two major and at least two minor proteins can be separated by electrophoresis in starch gel and on cellulose acetate. Gel filtration and column chromatography systems are being used as possible methods to resolve some of the proteins found in mouse erythrocyte membranes.

The carbohydrate moiety of mouse stroma has not been studied. We have determined the sialic acid content of the stroma from six inbred strains of mice. The values range from 27.5 μg per milligram of protein in DBA/2 to 33.0 μg per milligram of protein in A.SW mice, with B10.D2, A/SN, A.CA, and RFM having intermediate values. Preliminary genetic tests indicated that there was no association between sialic acid content and H-2 serotype. It was found, however that the level of sialic acid in serum showed an inverse relationship to the level of sialic acid in stroma. Sialic acid was the first carbohydrate studied because of its relevance to some blood group factors in man.

The lipids in the butanol phase of the water-butanol extract have not been studied.

Reference

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3.18 MUTATION INDUCTION OF SPECIFIC-LOCUS MUTANTS IN UV-SENSITIVE STRAINS OF *NEUROSPORA CRASSA*

F. J. de Serres

Recent studies by others[1–4] have shown that there are mutant strains of *Neurospora crassa* that are much more sensitive to inactivation following ultraviolet irradiation than standard wild-type strains. These mutant strains are assumed to have defective repair mechanisms, so that they have less efficient repair of the uv-induced genetic damage that produces inactivation. It is possible that these mutant strains also have less efficient repair of genetic damage that produces gene mutation and that these repair processes are specific for particular types of genetic damage. To investigate these possibilities a program has been started to induce *ad-3* mutants in these uv-sensitive strains.

Six different uv-sensitive strains will be studied: *uvs-1*[1], *uvs-2*[2], *uvs-3*[3], *uvs-4*[3], *uvs-5*[3], and *nuc-2*[4]. The effect of these six mutants on mutation induction at the *ad-3* loci will be tested first with haploid strains where only point mutations in the *ad-3* region can be recovered, and then with two-component heterokaryons (homozygous for a uv-sensitive marker) where both point mutations and chromosome deletion mutations in the *ad-3* region can be recovered.

Crosses have been made to incorporate each of the six uv-sensitive mutations into component II of heterokaryon 12[5] (a two-component heterokaryon heterozygous at the *ad-3A* and *ad-3B* loci) to obtain haploid strains marked at the *cot*, *al-2*, and *pan-2* loci. Although the six uv-sensitive mutants were induced in strains closely related to the St. Lawrence wild-type strain 74A, segregation has been observed among the progeny for factors affecting gross morphology, heterokaryon compatibility, and viability. Because of this a more extensive program of inbreeding has been initiated to ensure that these six uv-sensitive markers have a common genetic background.

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3.19 CHARACTERIZATION OF HEAVY-ION-INDUCED MUTATIONS IN THE *ad-3* REGION OF A TWO-COMPONENT HETEROKARYON OF *NEUROSPORA CRASSA*

F. J. de Serres

In a previous paper[1] we reported that both helium and carbon ions were more efficient than x rays in the production of mutations in the *ad-3* region of a two-component heterokaryon. For the overall induction of *ad-3* mutations, helium ions have an RBE of 1.8 and carbon ions an RBE of 5.0. Genetic characterization of the *ad-3* mutations induced by heavy ions is complete, and a comparison has been made with mutations in the *ad-3* region induced by x rays.[2] This comparison has shown that both heavy ions induce chromosome deletion mutations ($ad\text{-}3^{IR}$) more efficiently than point mutations ($ad\text{-}3^{R}$). For chromosome deletion mutations, helium ions have an RBE of 5.5 and carbon ions have an RBE of 73.8; for point mutations, helium ions have an RBE of 2.2 and carbon ions an RBE of 5.2.

The spectrum of chromosome deletion mutations induced by carbon ions is significantly different from that produced by x rays, but the spectrum of $ad\text{-}3^{IR}$ mutations produced by helium ions is not. With x rays the ratio of $ad\text{-}3A^{IR}$ to $ad\text{-}3B^{IR}$ to $(ad\text{-}3A\ ad\text{-}3B)^{IR}$ mutations is 0.16:0.34:0.49. With carbon ions the ratio is 0.21:0.49:0.30 with $P < 0.005$. With helium ions, however, this ratio is 0.15:0.32:0.52 with $P = 0.85$.

The spectrum of point mutations induced by carbon ions is also significantly different from that produced by x rays, but the spectrum of $ad\text{-}3^{R}$ mutations induced by helium ions is not. With x rays the ratio of $ad\text{-}3A^{R}$ to $ad\text{-}3B^{R}$ mutations is 0.36:0.66. With carbon ions this ratio is 0.45:0.55 with $P = 0.01$. With helium ions, however, this ratio is 0.30:0.70 with $P = 0.45$.

The spectrum of complementation patterns among the point mutations at the *ad-3B* locus induced by x rays is not significantly different from either the $ad\text{-}3B^{R}$ mutations induced by carbon ions or those induced by helium ions. The ratio of nonpolarized to polarized to noncomplementing mutants with x rays is 0.13:0.23:0.64. With carbon ions this ratio is 0.17:0.17:0.67 with $P = 0.40$. With helium ions this ratio is 0.09:0.28:0.02 with $P = 0.38$.

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3.20 GENETIC CHARACTERIZATION OF SPONTANEOUS MUTATIONS IN THE *ad-3* REGION OF A TWO-COMPONENT HETEROKARYON OF *NEUROSPORA CRASSA*

F. J. de Serres H. V. Malling
H. E. Brockman[1]

One of the important features of the *ad-3* test system in a two-component heterokaryon of *Neurospora crassa* is the low spontaneous frequency of *ad-3* mutations. Early experiments showed that the spontaneous forward-mutation frequency was lower than 1×10^{-6} heterokaryotic survivors, and in mutation-induction experiments with x rays[2] and nitrous acid[3] there was little doubt that the *ad-3* mutations obtained were actually induced.

Little is known about the nature of spontaneous specific-locus mutations, primarily because of the difficulty of obtaining a sample of sufficient size but also because of the lack of simple tests to characterize them genetically. Because the recessive lethal mutations at specific loci in the *ad-3* region of a two-component heterokaryon can be characterized by a series of simple genetic tests,[2,3] we have been trying to build up a sample of sufficient size for such a characterization since 1962. In experiments 12-1 through 12-94 on heterokaryon 12, a total of 172 spontaneous purple colonies have been recovered in the untreated control portions of the experiments. The average spontaneous forward-mutation frequency of purple colonies observed is 0.395×10^{-6} heterokaryotic survivors.

In the genetic analysis of these 172 mutants, both point mutations and chromosome deletion mutations were found. Among the 141 point mutations, 41 were $ad\text{-}3A^{R}$ and 101 were $ad\text{-}3B^{R}$. Among the 25 chromosome deletion mutations 6 were $ad\text{-}3A^{IR}$, 15 $ad\text{-}3B^{IR}$, and 4 $(ad\text{-}3A\ ad\text{-}3B)^{IR}$. The one $ad\text{-}3A^{IR}\ ad\text{-}3B^{R}$ double mutant was classified as both an $ad\text{-}3A^{IR}$ mutant and an $ad\text{-}3B^{R}$ mutant. A total of 6 of the 172 mutants could not be characterized because of excessively leaky growth. Heterokaryon tests for allelic complementation among the $ad\text{-}3B^{R}$ mutations gave a total of 53% (53/101) complementation, 41% (41/101) with nonpolarized patterns and 12% (12/101) with polarized patterns. A total of 47% (47/101) of the mutants were noncomplementing. Because of the high correlation between complementation pattern and

genetic alterations found among nitrous acid–induced[4] and ethyl methanesulfonate–induced[5] *ad-3B* mutants, we can conclude that a minimum of 41% of the *ad-3*^{R} mutants have resulted from missense mutations.

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3.21 GENETIC ANALYSIS OF *ad-3* MUTANTS OF *NEUROSPORA CRASSA* INDUCED BY ETHYLENE DIBROMIDE – A COMMONLY USED PESTICIDE

F. J. de Serres H. V. Malling

Ethylene dibromide (1,2-dibromoethane or EDB) is a broad-spectrum pesticide used against nematodes, fungi, and insects. EDB is very resistant toward microbiological and spontaneous breakdown in the soil. When used as a fumigant on tropical fruit, EDB can be found in the fruit when it reaches the market.

We have tested EDB for its mutagenicity in *Neurospora crassa* using the direct method[1] for screening for purple adenine mutants (*ad-3*). Conidia from a two-component heterokaryon were treated 3 hr with various concentrations of EDB: 1.2 to 1.63 μl per milliliter of a 0.06 *M* phosphate buffer adjusted to pH 7.0 containing 10% dimethyl sulfoxide (DMSO). In total 1139 *ad-3* mutants were isolated after this treatment with EDB; 7 mutants were isolated after treatment with DMSO alone.

The mutants are in the process of being analyzed genetically to distinguish point mutations from chromosome deletions and to obtain a presumptive identification of the genetic alterations in the point mutations at the molecular level.

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3.22 CAPTAN: A POTENT FUNGICIDE WITH MUTAGENIC ACTIVITY

H. V. Malling F. J. de Serres

Captan is a fungicide used in agriculture on crop plants of economic importance. The compound has been considered essentially "safe" for human beings, because the LD_{50} dose in rats and mice is high compared with other similar pesticides. Captan reacts almost exclusively with -SH groups in protein, and this selective reaction is most likely the basis for its toxic effect on fungi. Captan has teratogenic effects on chicken embryos, and it can induce chromosome aberrations. We have tested the compound on *Neurospora crassa* in a series of pilot experiments for its ability to induce (1) forward mutations in the *ad-3* region of a two-component heterokaryon and (2) reverse mutations in a series of tester strains whose mode of reversion represents the most common mutational mechanisms.

The forward mutations are induced in the heterokaryotic fraction of conidia of a two-component heterokaryon between biochemically different haploid strains. The *ad-3* mutants are selected by the direct method and can be either point mutations or multilocus deletions (chromosomal deletions). We found that Captan induced *ad-3* mutations in the forward-mutation system but not as yet in the reverse-mutation system. Genetic characterization of the Captan-induced mutants should elucidate the type of mutations induced by this compound.

3.23 PURIFICATION AND PROPERTIES OF PHOSPHORIBOSYL-AMINOIMIDAZOLE-SUCCINOCARBOXAMIDE SYNTHETASE FROM *NEUROSPORA CRASSA*

C. R. Fisher

An assay for the enzyme phosphoribosyl-aminoimidazole-succinocarboxamide synthetase [5′-phosphoribosyl-4-carboxy-5-aminoimidazole: L-aspartate ligase (ADP), EC 6.3.2.6] has been developed. This allows us to follow the enzyme through various purification routines and measure the recovery of enzymic activity at each step. The original purification procedures have been considerably modified. Present procedures were selected for suitability for bulk operations and for improved recovery of enzymic activity.

Current procedures involve ammonium sulfate fractionation, Sephadex G-50 and G-200 preparative

chromatography, and electrophoresis. Although purity data are not available at this time, enzymic activity is associated with a single well-separated symmetrical protein band after electrophoresis. Preliminary molecular weight studies by density gradient centrifugation indicate that the enzyme has a molecular weight of 25,000 to 30,000.

3.24 ANALYSIS OF COMPOUNDS USEFUL AS PROTEIN STAINS

C. R. Fisher

One of the major problems with electrophoresis is the locating of protein bands without denaturing the protein and without costly uv scanning equipment. One compound has been reported which causes protein bands to fluoresce under a uv source and which does not denature the protein. An attempt to obtain this compound, the magnesium salt of anilinonaphthalene sulfonate, showed that it was no longer available commercially. Eastman Organic Chemicals agreed to supply gratis a sample of extremely pure magnesium salt and also the sodium salt and free acid form of this compound. Tests using *Neurospora* proteins showed that the magnesium salt is superior to the other two forms, with the sodium salt and the free acid following in that order. The magnesium salt has once again been made available commercially by Eastman. Arrangements have been made to allow a further study of other compounds likely to have similar or superior properties.

3.25 MUTAGENICITY OF ACTINOMYCIN D

C. R. Fisher H. V. Malling

Actinomycin D is a very widely used compound. Its primary importance has been as an agent to stop DNA-dependent RNA synthesis. We have found that it is also a mutagen. Until recently only external binding of actinomycin to DNA had been reported. Now, however, based upon equilibrium, hydrodynamic, and kinetic studies it is indicated that actinomycin must also intercalate into the DNA molecule. Mechanisms can easily be visualized whereby the intercalation of the planar actinomycin molecule could occur and whereby such intercalation could lead to mutagenesis.

Based upon this information, *Neurospora* cultures were grown on medium containing actinomycin and on medium without actinomycin. After ten days of growth the conidia were harvested and subjected to the standard *ad-3* mutant isolation procedure. The number of mutants found after treatment with actinomycin D during the mitotic cycle is significantly greater than among the controls. There is no evidence that there is any additional effect during the meiotic cycle. There is some evidence of strain variation in sensitivity to actinomycin-induced mutagenesis.

3.26 A PURPLE STRAIN OF *NEUROSPORA* WHICH HAS NO ADENINE REQUIREMENT

A. J. F. Griffiths

Purple pigmentation is used as a selective identification tag for *ad-3* mutants. One such purple strain, 3-10-504, has, however, been found to show a linear growth rate on minimal medium which is equal to that of wild types. A genetic analysis of 3-10-504 in crosses to *ad-3* mutants has indicated that the genetic lesion responsible for its phenotype is closely linked to the *ad-3* locus but is not necessarily homologous to it. Investigations are in progress to determine the precise site of the lesion and if possible to discover the biochemical basis for the unusual phenotype.

3.27 GENETIC SIZE OF THE X REGION IN THE *ad-3* COMPLEX OF *NEUROSPORA CRASSA*

A. J. F. Griffiths

The *ad-3A* and *ad-3B* loci of *Neurospora* lie adjacently on chromosome I, separated by an area known as the X region. The presence of the X region is inferred both by genetic mapping in A × B crosses and from the fact that some complementation tests are interpretable only if a multicomplon region of unknown function is present between A and B. From an analysis of 70 A × B crosses, it has been established that the X region is genetically considerably smaller than previously believed (0.02 map unit) and that the A region is not less than 0.05 and the B not less than 0.07 map unit.

3.28 A STRAIN OF *NEUROSPORA* SHOWING ABNORMAL RECOMBINATION BEHAVIOR

A. J. F. Griffiths

A3 is an *ad-3A* strain which shows abnormally low recombination frequencies in the area of the *ad-3* locus on chromosome I. Up to twentyfold decreases have been encountered, compared with crosses using regular *ad-3A* strains. Furthermore, A3 shows extremely high negative interference in the three marked regions investigated on chromosome I. Experiments are under way to decide if the genetic factor responsible for these

effects is linked or unlinked to the area affected and also to determine the extent of the area of the genome affected.

3.29 GENETIC STUDIES OF THE SUPPRESSOR OF SABLE LOCUS OF *DROSOPHILA*

E. H. Grell

Mutations at the suppressor of sable locus, *su(s)*, suppress certain alleles of some mutants. The phenotype of *su(s)* includes an alteration of the tyrosyl-tRNA profile on RPC-2 columns (see Twardzik and Jacobson, *Biol. Div. Ann. Progr. Rept. Dec. 31, 1968,* ORNL-4412). Since this locus is the only one known to affect tRNA in a higher organism, genetic studies were begun in order to supplement the biochemical investigation.

Cytological Location of *su(s)*. – A series of X chromosome fragments bearing the tip of the X chromosome were tested for the presence of $su(s)^+$. Fragments including the 1B10-11 salivary chromosome bands carried $su(s)^+$, and fragments without those bands did not carry $su(s)^+$. The loci of $su(s)^+$ and $M(1)B1d^+$ were not separated in the tests of these fragments.

New Point Mutations of *su(s)*. – Two mutations of *su(s)* were recovered after EMS treatment and one after x irradiation of flies. No differences were observed which distinguish them from each other or from $su(s)^2$. They all suppress *v*, *pr*, and *sp*, and all cause flies to be less vigorous than normal. One of the EMS-induced alleles was tested by Twardzik and found to have the abnormal tyrosyl-tRNA profile like the original stock of $su(s)^2$.

Interaction of *su(s)* **and** *su(Hw)*. – $Su(Hw)^2$ is another mutation that suppresses a set of mutants different from those suppressed by *su(s)*. By itself it has no effect on the vermillion mutant (*v*), which is suppressed by *su(s)*. It was observed that $su(Hw)^2$ partially reverses the suppressive action of *su(s)* on *v*. This suggests that it would be of interest to examine profiles of tRNA's from $su(Hw)^2$.

3.30 TEMPORAL AND REGIONAL SPECIFICITIES OF OPPOSING EFFECTS OF TEMPERATURE ON CROSSING-OVER

Rhoda F. Grell

Whether or not crossing-over and DNA replication are intimately associated has not been resolved. One approach to this question in eucaryotes has been to study the relationship of the two processes in time. In *Drosophila* a system has been developed using immature females to determine the time of temperature-induced increase in crossing-over in the oocyte. The system permits the recovery of a well-synchronized population of eggs representing predominantly the first oocytes to undergo maturation. For this sample of oocytes the temperature effect in a 30-unit segment of chromosome 2 was found to be restricted to 126 to 162 hr of female development, with maximal increases occurring during the 18-hr period between 138 and 156 hr. Incorporation of ^{3}H-thymidine for the same sample of oocytes was demonstrated at 138, 144, and 150 hr but may include earlier and later times as well.

The temperature response of the entire X chromosome has now been examined. Increases were observed for distal and proximal regions only. The increases for the distal and the most proximal region were confined to 126 to 150 hr and were maximal for a temperature treatment between 132 and 144 hr. This maximum clearly precedes that found for chromosome 2 by approximately 12 hr. In marked contrast to this effect, conspicuous decreases were observed for the interstitial regions. The decreases occurred very early, between 114 and 132 hr, and here crossing-over was minimal for a temperature treatment between 120 and 132 hr.

The two opposing effects of temperature may be acting on two different components of the meiotic system. The early decrease, between 114 and 132 hr, would appear to coincide with the premeiotic anaphase or telophase and the initial formation of the oocyte; it may reflect an effect on exchange pairing. The later increase between 132 and 150 hr must coincide with the early premeiotic interphase and may be on the exchange process itself.

3.31 DISTRIBUTIVE PAIRING IN TRANSLOCATION HETEROZYGOTES

Rhoda F. Grell

Tests of the distributive model have now been carried out utilizing a wide variety of genetic situations. As predicted by the theory, exchange between homologs has been found to remain essentially unchanged regardless of the degree to which one of the homologs segregates from a nonhomolog and despite the restriction of nonhomologous associations to noncrossover chromosomes. A favorable test situation which hitherto had not been adequately explored is one utilizing translocation heterozygotes. Rearrangements of this kind, which are known to segregate from a nonhomolog with high frequencies, permit a determination of

crossover values throughout the length of the rearranged chromosome. A study of such chromosomes has now been completed.

A number of translocations between the right arm of chromosome 3 and the base of chromosome 4 were employed. In each case a larger element, T_3, carrying the left arm, centromere, and part of the right arm of 3 plus the euchromatin of 4, and a smaller element, T_4, carrying the centromere of 4 plus the remainder of 3R, had been produced. The translocations were chosen to constitute a series in which the break in 3R became progressively more distal, so that T_4 became progressively smaller. Some of the females carried a marked Y chromosome, while sisters of otherwise identical genotype lacked the Y. Genetic studies included (1) measurement of exchange in T_3 and T_4 for both XX and XXY females and (2) measurement of segregation between the marked Y and the translocation in the XXY females. Calculation of the frequencies of noncrossover tetrads (E_0's) for T_3 and T_4 were made from the crossover data and of Y,T association frequencies from the segregation data.

The crossover values and E_0 frequencies were found to be the same for XX and XXY females for each translocation studied. Percent crossing-over (and E_0's) for T(3:4)86D were XX = 96.6 (E_0's = 24.2), XXY = 93.8 (E_0's = 25.7); for T(3:4)89E they were XX = 68.6 (E_0's = 72.3), XXY = 70.4 (E_0's = 68.2); for T(3:4)A2 they were XX = 55.4 (E_0's = 98.4), XXY = 56.8 (E_0's = 96.8). Percent segregation of the translocations and the Y (and T,Y association frequencies) were Y,T(3:4)86D = 62 (ass. =24); Y,T(3:4)89E = 84 (ass. = 68); Y,T(3:4)94A2 = 93 (ass. = 86).

This test demonstrates once again, in a particularly unambiguous and decisive way, that crossing-over between homologs is unaffected by the participation of one member in high frequencies of association with a nonhomolog. Furthermore, as seen from a comparison of E_0 frequencies and T,Y association frequencies, virtually all translocation chromosomes which fail to undergo exchange enter into an association with the Y. These results can only be interpreted to mean that homologous and nonhomologous pairing are not competitive processes, that homologous pairing without exchange is insufficient to hold chromosomes together for segregational purposes until anaphase I, and that following exchange noncrossover chromosomes are free to enter into a second pairing, that is, distributive pairing, which provides the mechanism for their segregation.

3.32 STERILITY AND LETHALITY IN THE XYY MALE OF *DROSOPHILA MELANOGASTER*

Rhoda F. Grell

To examine the effect of two Y chromosomes on male fertility and viability, single Y's were extracted from the Canton-S, the Oregon-R, the Samarkand, and the Swedish-C strains of *D. melanogaster* and introduced into the same isogenic line. From these four lines, designated $Y^{C\text{-}S}$, Y^{Or}, Y^{Sam}, Y^{Sw}, a variety of XYY males with equivalent genetic backgrounds were synthesized. Each type carried one of the ten possible pairwise YY combinations as well as a Canton-S X chromosome with proximal heterochromatin from one of the same four strains, designated $X^{C\text{-}S}$, X^{Or}, S^{Sam}, X^{Sw}. In all 29 different XYY genotypes were studied.

Certain YY combinations were found to confer sterility or semisterility upon the male. Sterility tended to be associated with the presence of two identical Y's rather than with different Y's. Thus, $Y^{C\text{-}S}$ $Y^{C\text{-}S}$ with any of the four X bases and Y^{Sam} Y^{Sam} with $X^{C\text{-}S}$ or X^{Sw} exhibited complete or near complete sterility; Y^{Or} Y^{Or} with X^{Or} or X^{Sw} semisterility; Y^{Sw} Y^{Sw} with X^{Or} or X^{Sw} reduced fertility. The only combination of different Y's showing sterility was $Y^{C\text{-}S}$ Y^{Sam} when accompanied by an X^{Or} or X^{Sam}. Other YY combinations were found to be lethal or semilethal, since males carrying them were never or rarely recovered. These included Y^{Sam} Y^{Sam} with X^{Or} and $Y^{C\text{-}S}$ Y^{Sam} with $X^{C\text{-}S}$ or X^{Sw}.

These results demonstrate that Y chromosomes within the *melanogaster* species are not necessarily genetically equivalent. An extra dose of one or more genes present in particular Y's can lead to sterility or lethality. In the human species the Y chromosome, morphologically at least, displays a measurable degree of diversity. The human XYY male also exhibits marked variability in fertility, mental capacity, and certain physical aspects. The phenotypic variability found in the human XYY male may, as in *Drosophila,* have its origin in the particular combination of Y chromosomes present.

3.33 THE ROLE OF SIZE IN DISTRIBUTIVE PAIRING OF LARGE CHROMOSOMES IN THE *DROSOPHILA* GENOME

Charleen M. Moore[1]

Noncrossover chromosomes in *Drosophila* females participate in a process known as distributive pairing,

which is regulated by chromosome size. Two noncrossover chromosomes segregate from each other quite regularly (~90–99%). With three elements of different sizes, the tendency is for the intermediate element to move toward one pole while the larger and smaller elements move to the other. The tendency is strongest when the size of the intermediate element approaches the geometric mean of the other two. If two of the elements are similar in size and the third is markedly dissimilar, the similar elements segregate regularly, while the dissimilar one assorts randomly.

These rules were derived primarily from studies of small chromosomes, the largest being about half the size of a normal X. The present study has as its purpose to determine whether the larger chromosomes of the *Drosophila* genome follow these same rules. To do this, compound chromosomes, that is, two homologs attached to a single centromere, were utilized. A compound X ($\overline{XX}$), which is a very large chromosome, and a compound 4 ($\overline{44}$), which is a small chromosome, were introduced into a female carrying one of a series of X duplications (Dp). These are deleted X's possessing only a small distal euchromatin tip and a variable amount of proximal X heterochromatin. The X duplications ranged in size from approximately 0.3 to 4+ times the size of a single fourth chromosome, as measured at mitotic metaphase in brain cells. Depending on the duplication used, it constituted the small or intermediate element with the $\overline{XX}$ and $\overline{44}$.

All the duplications showed nondisjunction frequencies in excess of 50%. It must be assumed from this that in all cases trivalents are being formed and that chromosome segregation from these trivalents is nonrandom. For the Dp's of size 0.3 to 1.4, $\overline{XX}$,Dp nondisjunction frequencies ranged from 74.1 to 80.6%, indicating that the intermediate element ($\overline{44}$) was directing the larger ($\overline{XX}$) and smaller (Dp) elements to one pole while it moved to the other. For the Dp of size 4+, however, the $\overline{XX}$,$\overline{44}$ nondisjunction frequency was 53.4%. In this case the Dp became the intermediate element directing the $\overline{XX}$ and $\overline{44}$ to one pole while it moved to the other. Dp's between sizes 1.4 and 4+, which are now being tested, will indicate the exact point at which this shift occurs. The evidence thus far indicates that the larger chromosomes in the *Drosophila* genome follow the same pairing rules as described for the smaller ones.

Earlier work has shown that small chromosomes which segregate regularly in the oocyte also undergo nonhomologous pairing in the oogonial cell, but to a lesser degree. A cytological examination of the oogonial cells at metaphase has been started. If nonhomologous pairing is found with these genotypes, a study of somatic cells will be carried out to determine whether or not such pairing is restricted to the germ cell line.

Reference

[1] Predoctoral investigator.

3.34 STRUCTURE AND FUNCTION OF MULTIENZYME COMPLEXES

F. H. Gaertner K. W. Cole

This group was established during the current year and is only recently beginning experimental work. Specific problems to be investigated by this new group include: mechanism of catalytic facilitation by multienzyme complexes;[1] structure and catalytic properties of enzymes and multienzyme complexes in the aromatic-tryptophan-NAD pathway of *Neurospora crassa*;[2,3] nature and extent of compartmentation of biochemical intermediates by multienzyme complexes; possible role of membranes in the organization and function of the enzymes catalyzing the aromatic-tryptophan-NAD pathway; comparative biochemistry of the enzymes catalyzing the NAD pathway of mammals and *N. crassa.*

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3.35 FURTHER OBSERVATIONS ON THE STRUCTURE OF ACTIVE NUCLEOLAR GENES

O. L. Miller, Jr. Barbara R. Beatty[1]

Preliminary characterization by electron microscopy of redundant nucleolar genes actively synthesizing rRNA precursor molecules was reported last year.[2] By using preparative procedures which stretch such genes we have succeeded in visualizing individual RNA polymerase molecules during transcription (Fig. 3.35.1). The enzyme molecules typically appear as well-defined nearly spherical granules about 125 A in diameter. Granules similar in size and electron density often are present on the inactive segments which separate the nucleolar genes. Since each gene appears to be completely loaded with RNA polymerase molecules

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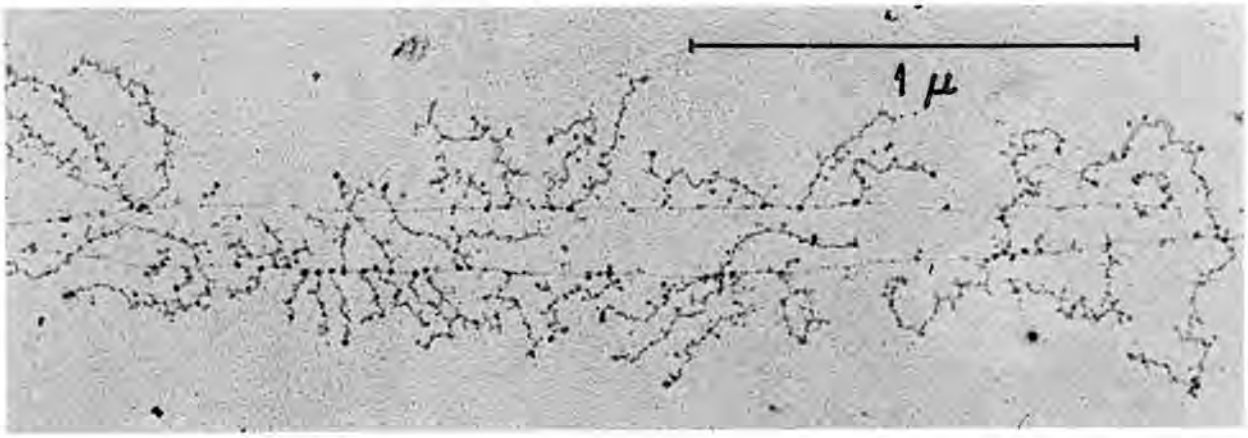

Fig. 3.35.1. Portion of a Highly Stretched, Active Nucleolar Gene. RNA polymerase molecules are seen as dense, spherical granules located on the gene axis. RNP fibrils containing rRNA precursor molecules in various stages of maturation are attached to the enzyme molecules. Scale line = 1 μ.

at all times, this raises the possibility that the spacer regions are staging areas for polymerase molecules to ensure high efficiency of transcription. This suggestion could be tested if a ferritin-tagged antibody to the polymerase could be obtained.

Estimates of the molecular weight of completed precursor molecules range between 2.5 and 3.0 × 10^6 daltons. Fully extended, such molecules would be between 5 and 6 μ long. The RNP fibrils attached to polymerase molecules at the termination end of each gene are ~0.5 μ long. Consequently, mature precursor molecules must be foreshortened in some manner by their protein coats to give a 10 to 12:1 ratio of RNA to RNP fibril length.

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3.36 FINE STRUCTURE OF BACTERIAL GENES IN ACTION

O. L. Miller, Jr. Barbara A. Hamkalo[1]
C. A. Thomas, Jr.[1]

Techniques developed for visualizing the structure of active genes in a eukaryotic cell[2] were used to observe chromosomes of *Escherichia coli* isolated from cells in log growth phase. A mutant strain, which under certain growth conditions develops fragile cell walls, was utilized.[3] Fragile cells were osmotically burst by rapid dilution into distilled water and then centrifuged onto support films and stained for electron microscopy.

Previous biochemical studies of the fragile strain showed that all mRNA molecules are associated with ribosomes in the cell.[3] Our preliminary observations indicate that, in addition, all mRNA molecules are attached to the *E. coli* chromosome (Fig. 3.36.1). Each messenger is attached to the chromosome at the site of an irregularly shaped granule about 75 A in diameter. We conclude that the granules are RNA polymerase molecules which were actively transcribing genes at the time of isolation. Messengers typically appear to be saturated with ribosomes, and the first ribosome to attach to an mRNA molecule translates immediately behind the RNA polymerase molecule. The speed of translation thus appears to be controlled by the rate of transcription. It has been estimated that the completed messenger of the five-gene tryptophan operon of *E. coli* has about 100 ribosomes associated with it.[4] The largest polyribosome so far observed in these studies contained 40 ribosomes.

The spacing of polyribosomes on single genes or operons is irregular, but the average spacing when several genes are combined is about 0.2 μ. The approximate location of the initiation of messenger synthesis sometimes can be inferred from the gradient of polyribosome sizes. Such sites often appear to be

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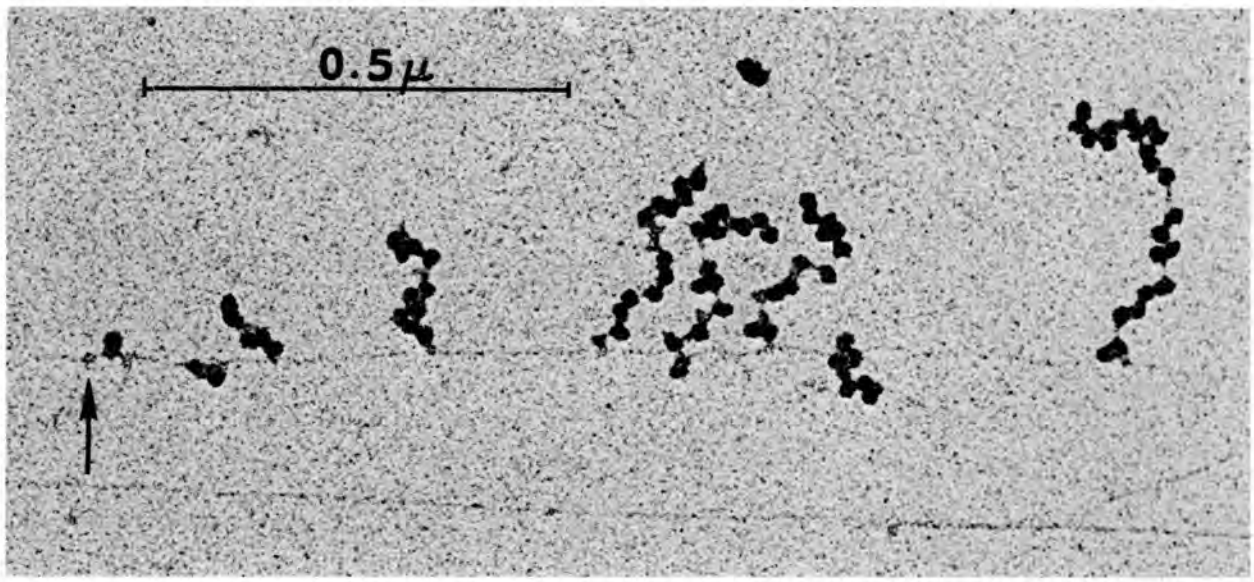

Fig. 3.36.1. Genetically Active and Inactive Portions of Chromosome Isolated from a Fragile Strain (N181) of *E. coli.* The polyribosomes attached to the active segment exhibit an imperfect gradient of increasing lengths. The arrow indicates a presumptive RNA polymerase molecule on or very near the initiation site of the gene or operon.

PHOTO 98867

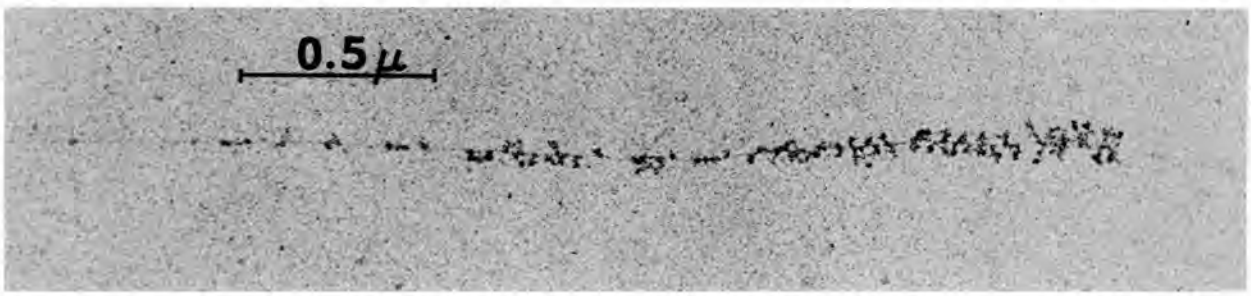

Fig. 3.36.2. A Presumptive rRNA Gene in an *E. coli* Chromosome.

occupied by RNA polymerase molecules. Although the termination points of active loci cannot be determined, the distance between a presumptive initiation site and the most distant polyribosome has ranged between 0.33 and 3.0 μ for the loci observed so far. The number of polyribosomes per active locus usually is correlated with the length of the locus as estimated by this method. Polyribosome configurations suggesting convergent and divergent gene polarities as well as similar polarities have been observed.

Estimates of length of messengers in polyribosomes can be made when the initiation site of a gene can be approximated. For genes which do not appear to have been stretched by the preparative procedures, the ribosomes, which are 200 to 250 A in diameter, appear to be spaced about 500 to 1000 A along the messenger molecules.

Much of the *E. coli* chromosome does not have attached polyribosomes, indicating that many genes are completely repressed at any one instant during cell growth. Numerous granules similar to active RNA polymerase molecules in size and electron density are present on the "silent" segments of the chromosome. The chromosome itself is about 40 A in diameter, suggesting that the duplex DNA molecule is coated with another component (basic protein?).

Various estimates of the redundancy of rRNA genes in *E. coli* suggest the presence of five to ten each of the

genes coding for the 16S and 23S rRNA's per chromosome. Other studies have indicated that each of these genes must be transcribed simultaneously by a relatively large number of RNA polymerase molecules[5] and that some ribosomal protein becomes associated with the rRNA's as they are synthesized.[6] Chromosomal segments exhibiting 60 to 70 attached fibrils have been observed (Fig. 3.36.2). When unstretched, the length of these segments is close to the length of DNA necessary to code for one 16S and one 23S rRNA molecule (~1.65 μ DNA). We suggest that these segments are the rRNA genes; if so, these redundant genes do not appear to be closely clustered on the chromosome.

Future research using these techniques will be directed toward identifying specific genes in action on episomes as well as in the chromosome and visualizing viral integration into the bacterial genome.

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3.37 A CULTURE PROCEDURE FOR THE STUDY OF PROTEIN INCORPORATION BY AMPHIBIAN OOCYTES

R. A. Wallace D. W. Jared

Previous studies performed primarily on intact *Xenopus* have indicated that large amounts of protein are sequestered from the blood by hormonally activated oocytes during yolk formation. A serum protein, designated vitellogenin, has also been found to be incorporated at least 25 times more rapidly than other serum proteins tested, and it apparently serves as a direct precursor to the yolk proteins phosvitin and lipovitellin. The factors involved in the uptake of proteins by vitellogenic oocytes are difficult to study using the intact animal, so we have accordingly developed a method for the culture of isolated developing oocytes. The most suitable in vitro conditions appear to involve a relatively high protein content together with a simple hypotonic salt solution: 50% *Xenopus* serum (containing vitellogenin) thoroughly dialyzed against 90 m*M* NaCl, 1.5 m*M* KCl, 1 m*M* $CaCl_2$, and 4 m*M* Tris-Cl, pH 8.0. When individual oocytes within the size range 0.8 to 1.1 mm were isolated, divested of their outer membrane (which is impermeable to protein), and subsequently cultured in the above medium containing the standard antibiotics, labeled serum protein was incorporated by the oocytes as a linear function of time for at least six days. Protein uptake was also 15 times more rapid in oocytes derived from vitellogenic females than in oocytes from nonvitellogenic females. Radioautographs of the cultured oocytes indicated that virtually all the incorporated protein became associated with the interior of the oocyte and did not simply accumulate at the surface.

It would thus appear that the initial conditions have been met for the use of the developing amphibian oocyte as a model system for the study of hormonal and other factors controlling protein incorporation at the cell membrane. We are presently studying the effects of donor history (after hormone treatments), follicular membranes, and oocyte size on the extent of protein uptake and have found that the selective mechanism for vitellogenin incorporation also operates in the isolated oocyte.

3.38 OOCYTE DEVELOPMENT IN *XENOPUS LAEVIS*

J. N. Dumont M. Virginia Cone

Studies of oogenesis in various organisms have contributed significantly to an understanding of how cells interact, both with each other and with their extracellular environments. The intricate events associated with oocyte development in *Xenopus laevis*, for example, are not necessarily confined to the oocyte itself, but also occur in, and are subject to regulation by, other parts of the organism.

Very early studies of yolk formation in amphibians focused on events occurring within the oocyte; active roles in vitellogenesis were attributed to nearly every cellular organelle. Only in recent years has the extraoocytic origin of deutoplasmic substances been conclusively demonstrated.[1]

Previous investigations led their authors to conclude that vitellogenesis occurred in oocytes which we *now*

consider are, in fact, *pre*vitellogenic. Thus, reexamination of these early oocytes seemed necessary for greater understanding of the later, vitellogenic, stages, as well as oocytic and extraoocytic regulation of yolk formation.

The ultrastructural characteristics of previtellogenic oocytes, those less than approximately 0.3 mm in diameter, are strikingly different from those of larger, vitellogenic, oocytes. In previtellogenic oocytes, the germinal vesicle is centrally located. Large quantities of electron-dense material, presumably ribonucleoprotein, appear to be entering the perinuclear ooplasm via the nuclear pores. There is evidence that a portion of this material may become associated with the "vitelline body," a small region in which hundreds of mitochrondria are concentrated.

The ooplasm of these early stages exhibits no ultrastructural evidence of vitellogenic activity. There are a few elements of endoplasmic reticulum, but numerous profiles of Golgi systems are present. Relatively dense membranous inclusions reminiscent of mitochondria lie, often in clusters, in the cortical ooplasm. Also seen are granular bodies which, in later stages, condense and perhaps fuse to eventually form the definitive pigment of the maturing oocyte.

Previous studies in this laboratory have shown that micropinocytosis is directly related to the accumulation of yolk. Although this mechanism is relatively specific for the uptake of yolk precursor, other materials are also incorporated. The mature yolk platelet is a bipartite structure consisting of a crystal surrounded by a homogeneous superficial layer. The uptake and fate of heterologous nonyolk materials were monitored by using the following tracers: ferritin, iron dextran, peroxidase, Thorotrast, and colloidal gold.

Within 24 hr, the electron-opaque tracers peroxidase, ferritin, and iron dextran are found in the spaces between the various cellular and connective tissue layers surrounding the oocyte, as well as in micropinocytotic pits and vesicles in the periphery of the oocyte. After 48 hr, the tracers are located in spherical preplatelets situated in the cortical ooplasm. At this stage there is no indication of crystal formation within these structures; however, after several days the preplatelet bodies have fused with each other or with small yolk platelets, and crystal formation has begun. Tracer materials are never included within the crystal, but are confined to the superficial layer of the platelet. In these cases the superficial layers are considerably larger than those of platelets in oocytes that were not exposed to the tracers. Thus, both the tracer material and yolk protein precursor are incorporated by the oocyte, but the tracer materials never appear in the crystalline portion of the platelet. The results of the Thorotrast and colloidal gold experiments are inconclusive, but preliminary evidence suggests that they never reach the surface of the oocyte. Such "filtration" may partially account for the specificity of SLPP uptake since other substances may be prevented from reaching the oocyte by the same mechanism.

The use of these tracers has provided evidence that, despite the selective uptake of SLPP, other materials are nonselectively incorporated. Since these materials are confined to the superficial layer of the platelet, it is concluded (1) that segregation of some nonyolk proteins takes place within the spherical yolk bodies, probably through the process of crystallization, and (2) that the superficial layer is composed of substances other than specific yolk proteins.

Many cytological and physiological events associated with gametogenesis in female *Xenopus* are initiated and/or regulated hormonally. Investigation of one of these, melanogenesis, is currently being studied and is the subject of another report.[2]

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3.39 THE EFFECTS OF TOYOCAMYCIN ON RNA SYNTHESIS IN *XENOPUS* OOCYTES

Kathryn J. Kull[1] J. N. Dumont M. Virginia Cone

Biochemical evidence suggests that toyocamycin selectively inhibits the synthesis of ribosomal RNA in mammalian cells by preventing the conversion of 45S precursor into 28S and 18S.[2] Experiments were designed to examine the effects of toyocamycin on RNA synthesis in *Xenopus* oocytes. Small pieces of ovary were removed from animals which had been injected seven days previously with human chorionic gonadotrophin (HCG). The tissue was incubated in amphibian Ringer's containing 0.01 and 0.005 μg/ml of toyocamycin for 30 min prior to the addition of ^{3}H-uridine. Incubations were continued for 2, 8, 14, and 21 hr, at which times pieces were removed, fixed in Carnoy's fluid, and prepared for autoradiography. Controls were treated in the same manner except that toyocamycin was omitted. The autoradiographs were examined and the grains per nucleolus were counted from each group. In all sizes of oocytes the grain counts were higher in the experimental than in the control,

indicating that toyocamycin causes an accumulation of the product in the nucleolus. In small (0.2- to 0.3-mm-diam) previtellogenic oocytes, the increase in grains per nucleolus is relatively slow during incubation. In larger vitellogenic oocytes, the rate is more rapid. Preliminary analysis of data relating to the ratio of grains in nucleoli to grains in the nucleoplasm suggests that, in the experimental groups, less material is transferred from the nucleoli into the nucleoplasm than in the controls. These results support the view that toyocamycin is effective in preventing the breakdown of nucleolar RNA and its subsequent transfer into the ooplasm.

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3.40 THE SYNTHESIS OF MELANIN PIGMENT IN THE OOCYTES OF *XENOPUS LAEVIS* AND THE EFFECTS OF THE TYROSINASE INHIBITOR PHENYLTHIOUREA (PTU)[1]

J. N. Dumont J. J. Eppig, Jr.[2]

These experiments were initiated for the purpose of examining (1) the fine structure of oocyte premelanosomes and their maturation into melanosomes, (2) whether PTU could be employed to inhibit the formation of melanosomes in oocytes, and (3) the relative activities of the melanin synthesizing enzyme, tyrosinase, in nonvitellogenic, vitellogenic, and PTU-treated vitellogenic oocytes.

Adult *Xenopus laevis* females were injected with human chorionic gonadotrophin (HCG) every other day to induce ovulation. They also received daily intraperitoneal injections of 2 ml of 0.4% PTU. Vitellogenic control animals were given identical HCG injections but received 2 ml of 0.1 *M* NaCl daily every day instead of PTU. Nonvitellogenic controls received no treatments. Ovarian biopsies were made 21 days after initiation of treatment. Some eggs were fixed for electron microscopy, while others were staged according to size into three groups (small, 0.1 to 0.5 mm; medium, 0.5 to 1.0 mm; large 1.0 to 1.2 mm) for tyrosinase assay.

While macroscopic and light microscope observations show that oocytes which develop in the PTU-treated animals are devoid of pigment, electron microscopic studies reveal that premelanosomes are formed but that melanin deposition is inhibited. These premelanosomes are membrane-bound spherical structures of varying diameters. Some contain particles about 200 A in diameter while others contain small parallel rodlike elements about 100 A in diameter. When these are examined in cross section they present a hexagonal or paracrystalline pattern and are thought to represent early stages of melanosome development. This pattern is infrequently seen in the normal oocyte, presumably because it is obscured by the dense deposits of melanin.

Radiometric tyrosinase assays were performed on the three stages of oocytes from the nonvitellogenic, vitellogenic, and PTU-treated vitellogenic females. This assay measures the amount of ^{14}C-tyrosine converted into melanin by tyrosinase.

These data show that the medium-size oocytes from the vitellogenic control animals have about ten times greater tyrosinase activity than comparable oocytes of the PTU-treated vitellogenic and the nonvitellogenic ovary. This suggests that HCG either directly or indirectly stimulates melanin synthesis in developing oocytes. The tyrosinase activities of the small- and medium-sized nonvitellogenic and the PTU-treated vitellogenic oocytes are essentially identical. However, the activity of the large oocytes from vitellogenic control animals is about 20% higher than that of the similar-size oocytes from nonvitellogenic animals.

When PTU treatment and induced ovulation are terminated, the developing oocytes again begin pigment deposition, but the larger nearly mature oocytes present in the ovary at this time do not. These large unpigmented oocytes are eventually ovulated and are capable of being fertilized and of developing into normal embryos. The embryos remain pigmentless until, at the time of hatching, they begin to synthesize their own melanin.

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[1] Research jointly sponsored by the National Cancer Institute, the National Institute of General Medical Sciences, the National Institute of Allergy and Infectious Diseases, and the U.S. Atomic Energy Commission under contract with Union Carbide Corporation.

[2] Oak Ridge Graduate Fellow from Catholic University of America, Washington, D.C.

3.41 THE DEVELOPMENT OF NUCLEOLI IN THE IRIS EPITHELIUM DURING THE INITIAL STAGES OF WOLFFIAN LENS REGENERATION

J. N. Dumont Tuneo Yamada M. Virginia Cone

The initiation of rRNA synthesis in the dorsal iris epithelium is one of the first biochemical events

associated with Wolffian lens regeneration in the newt, *Triturus viridescens.*[1] Since the precursor of rRNA is synthesized in the nucleolus, an examination of nucleolar changes in this regenerating system was made.

In normal dorsal iris epithelial cell the nucleoli are small compact structures composed almost entirely of a mass of 20- to 50-A fibers. Some, however, do have an associated granular component (100 to 200 A in diameter). Measurements made from electron micrographs indicate that 62% of the nucleolar area is occupied by the fibrous component, 9% by a granular region in which the granular component dominates and the fibrous component is insignificant or absent, and 29% by a fibrogranular region composed of both fibrous and granular elements.

Within two days following lens removal and the subsequent initiation of lens regeneration, the nucleoli increase in size. All nucleoli contain a large amount of granular as well as fibrous component. The fibrous region occupies about 33% of the total nucleolar area, the fibrogranular region about 44%, and the granular region about 23%. Portions of the fibrogranular region often form coarse threadlike extensions of the nucleolus.

Structurally, the nucleoli of the dorsal iris epithelium four days after lens removal are similar to those of the two-day regenerate. The percentage of the nucleolar area occupied by each region is similar to that of the two-day regenerate. During the first four days of lens regeneration the total area of the nucleolus increases about fourfold, from 0.75 μ^2 in the normal to about 3.0 μ^2 in the four-day regenerate.

In addition to these structural changes in the nucleolus during lens regeneration there is also an increase in the number of nucleoli. Nucleoli in squash preparations of dorsal and ventral iris were counted using phase-contrast optics. In the normal dorsal iris, slightly less than 25% of the cells contain nucleoli. This value doubles during the first two days after lentectomy and then remains essentially constant during the subsequent three days. The number of nuclei containing nucleoli also increases. For example, the number of binucleolate nuclei increases threefold in two days. A small percentage of the nuclei may contain three to six nucleoli.

Nucleoli also increase in number in the ventral iris epithelium, but this increase is usually slower and less dramatic than in the dorsal iris. In the normal ventral iris epithelium, the number of nuclei containing nucleoli is about 5% lower than in the dorsal iris. This difference increases at one day after lens removal and remains an average of 30% lower than the dorsal throughout the remaining four days. There is also a slight increase in the number of bi- and multinucleolate nuclei.

These nucleolar changes parallel the activation of rRNA synthesis in the iris tissue during the initial stages of Wolffian lens regeneration. Such changes may be directly related to the activation of rRNA synthesis and as such represent its morphological expression.

Reference

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3.42 IN VITRO ACTIVATION OF RIBOSOMAL RNA SYNTHESIS

D. H. Reese

The earliest event so far detected in the process of Wolffian lens regeneration is the activation of ribosomal RNA (rRNA) synthesis. This activation takes place in the cells of the dorsal iris within two days after lens removal and precedes the activation of DNA synthesis by at least two days. Because it occurs early during regeneration and is essential for growth and differentiation of the new lens, an understanding of the way in which it is controlled and how it influences other events is of prime importance in the analysis of Wolffian lens regeneration. We have found recently that normal iris tissue can be induced to synthesize rRNA by simply removing the tissue and culturing it.

If normal dorsal iris is removed from the animal and cultured in newt-adjusted Eagle's minimum essential medium containing 10% fetal calf serum, an increase in ^{14}C-uridine-labeled phenol-extracted RNA can be detected after the first day in culture. Sucrose density gradient centrifugation further reveals that much of the newly synthesized RNA sediments where the two cytoplasmic and rRNA components are expected to sediment. The rate of RNA synthesis continues to increase up to four days in vitro (longer culture periods have not been examined). Based on the total amount of labeled RNA, there is a 1.6-fold increase in rate of RNA synthesis after one day in culture, a 1.8-fold increase after two days in culture, and a 3.2-fold increase after four days in culture. Sucrose density gradients reveal that most (but not all) of this is rRNA. (A peak of radioactivity appears on the bottom of the gradient and another just above the bottom. The increase in the rate of synthesis of these two heavy non-rRNA components appears to parallel that of rRNA synthesis. There is also

strong indication that another non-rRNA species shows an increased rate of synthesis in vitro. This species sediments close to the lighter rRNA component.)

Evidence obtained so far indicates that this in vitro activation of rRNA synthesis is quantitatively and qualitatively equivalent to in vivo activation. The rate of total RNA synthesis of the four days in vitro and four days in vivo shows no significant difference. Their sucrose density gradient profiles are also the same. Furthermore, the number of nuclei showing activated nucleoli in dorsal iris activated in vitro is the same as that for in vivo activated tissue. Also, electron micrographs reveal that the nucleoli of in vitro activated tissue show the same typical "activated" state as in vivo activated tissue. This evidence for the increase in nucleoli number and change in nucleoli ultrastructure make it unlikely that the change in rate of RNA synthesis measured by the incorporation of ^{14}C-uridine is due to a change in uridine pool size.

3.43 THE ROLE OF THE MACROPHAGE IN DEPIGMENTATION OF IRIS EPITHELIAL CELLS

Tuneo Yamada J. N. Dumont Marion E. Roesel

During transformation of newt iris into lens, melanosomes which pack the cytoplasm of normal iris epithelial cells disappear, so that when these cells later start to accumulate lens crystallins the cytoplasm is free from melanosomes. The number of cell divisions induced during the process of tissue transformation is estimated to be 2 to 5 and is too small for dilution to be causing melanosome disappearance. On the basis of some electron microscopic observations, Eguchi came to the conclusion that the melanosomes of iris epithelial cells are actively removed by amoeboid cells, which become loaded with melanosomes during depigmentation. Our light microscopic observations of bleached and nonbleached serial sections of the iris in the normal condition and at various stages of Wolffian lens regeneration, together with electron microscopic observation of "amoeboid cells," have furnished the following information:

The "amoeboid cells" have an interphase nucleus with large heterochromatic areas and characteristic margination, cytoplasm containing lysosomes and rough-surfaced endoplasmic reticulum, and a large number of villi at the cell surface. The similarity of their structure to mammalian monocytes is striking. We tentatively designate these cells as macrophage. The interphase nucleus of macrophages can be easily distinguished by light as well as electron microscope from that of iris epithelial cells.

In the normal condition, the macrophages can be infrequently observed near to, applied to the surface of, and even within the iris epithelium. After lens removal their number increases sharply, and many of them penetrate into the iris epithelium between epithelial cells. However, they do not adhere to the latter cells. Thus no incorporation of macrophages into the epithelial arrangement can be observed. Inside the iris epithelium, macrophages become loaded with melanosomes. The macrophages, with their cytoplasm expanded with melanosomes, then migrate into the optic cavity, iris stroma, or blood capillaries. One can observe subsequent fragmentation of the macrophage nucleus and sharp reduction in the amount of melanosomes.

These observations indicate that macrophages phagocytose the melanosomes discharged by the iris epithelial cells, transfer them outside the iris epithelium, digest the melanosomes, and eventually degenerate. The possibility that the penetration of iris epithelium by macrophages specifically provides a stimulus for tissue transformation is ruled out by the fact that this penetration occurs not only in the dorsal margin of the iris epithelium, the only area of transformation, but over the whole epithelium, including the pars ciliaris. Also excluded is the possibility that macrophages participate in formation of the new lens, because the lens regenerate is formed from an epithelium containing nuclei of the iris epithelial type and not of the macrophage type.

3.44 THE PATTERN OF MITOSIS DURING INDUCED PROLIFERATION OF IRIS EPITHELIUM

Tuneo Yamada Marion E. Roesel

It was earlier demonstrated that removal of the lens from the adult newt eye leads to incorporation of ^{3}H-thymidine into nuclei of iris epithelial cells. However, for obtaining information about cell proliferation, data on mitosis are also needed. For a quantitative study of mitosis in the iris epithelium, the presence of melanosomes causes technical difficulties. Bleaching the pigment with hydrogen peroxide solves this problem. Complete serial sections of the irises of the normal adult newt and those of animals at 3, 4, 5, 10, 15, and 20 days after lens removal were bleached, stained with hemalum, and scanned for mitotic figures. The number of mitotic figures per dorsal, lateral, and ventral sector of pars iridica and those per pars ciliaris were determined, and the percentage of mitotic cells in total cells in the respective areas was computed.

In the normal and three-day series, no single mitosis was registered in any area. In the four-day series a very small number of mitoses were found in the dorsal pars iridica. At around five days the number and percentage of mitoses in all areas of the pars iridica increased and reached peak values around 10 and 15 days. In the pars ciliaris the frequency of mitosis remained very low during the whole period.

The details of the present data combined with earlier data on the distribution of labeled cells imply that the iris epithelial cells enter their first induced cell division after completing the first induced S phage, and that the majority of cells activated in DNA replication proceed to mitosis.

The recent observation that RNA synthesis is activated in the normal iris cultured in vitro raises the question whether under the same culture condition the normal iris epithelium will start to proliferate. Preliminary study indicates that proliferation is indeed strongly initiated. This observation opens up a new approach to the control mechanism of proliferation.

3.45 TYROSINASE ACTIVITY DURING WOLFFIAN LENS REGENERATION

R. K. Achazi

One of the striking features of Wolffian lens regeneration in the newt is depigmentation. After lens removal, cells of the dorsal margin of the iris epithelium discharge their melanosomes, start cell division, and finally form a new lens. We tried to follow the transformation by measuring the activity of tyrosinase, which is involved in the formation of melanin. This enzyme catalyzes the steps from L-tyrosine to 3,4-dihydroxyphenylalanine (dopa) and from dopa to dopaquinone. The assay therefore measures only the initial steps of the reaction sequence of melanogenesis and allows only a limited conclusion concerning the end product, the melanosome. However, a close correlation has been found between occurrence of melanosomes and tyrosinase in animal cells.

Tyrosinase activity per microgram of DNA is at a low level both in the dorsal and ventral irises of normal animals. The activity of both areas rises sharply after lens removal. By the fourth day after lens removal, both dorsal and ventral irises display an activity approximately four times their normal level. This high level of activity is still seen six days later. At this time the activity of the ventral iris is slightly lower than that of the dorsal iris. During the subsequent stages, when depigmentation of dorsal marginal iris becomes quite obvious, the enzyme level in the dorsal iris continues to rise, while it remains approximately unchanged in the ventral iris. Thirty days after lens removal the regenerated lens has separated from the dorsal iris. At this time the activity of the new lens reaches a level approximately 12 times that of the normal iris. After five months, when the regenerated lens is nearly as large as the normal lens, its activity is twice as high as that of the normal iris. Since the normal lens has activity closely comparable with that of the normal iris, the regenerated lenses of the two stages studied are distinguished from the normal lens by their higher enzyme activity.

When protein synthesis is inhibited with Actidione (cycloheximide) the data of our assay are not affected. This suggests that incorporation of labeled tyrosine measured in the present assay is not related to protein synthesis.

The data on the tyrosinase level are unexpected in the sense that a rise instead of a fall occurred during depigmentation and lens formation. Possible involvement of inhibitors and different pool sizes in our assay is being checked. Application of immunochemical methods to enzyme determination in this system is urgently needed. Efforts are being made to purify amphibian tyrosinase for obtaining specific antibodies.

3.46 TYROSINASE ACTIVITY DURING LENS DEVELOPMENT IN *RANA PIPIENS*

R. K. Achazi

In Wolffian lens regeneration the lens is formed from cells derived from iris epithelial cells which contain a huge amount of melanosomes. In normal development the lens is formed from head ectoderm cells, which in most amphibian species also contain melanosomes, but in a very low concentration. As reported elsewhere in this report, the lens regenerate shows a level of tyrosinase activity much higher than that of the normal lens. Since tyrosinase functions in melanogenesis, the above observation may indicate that regenerated lens cells retain the characteristic of iris cells, a high level of tyrosinase, even after transformation. This consideration has prompted us to study the tyrosinase activity in normal lens development.

Lenses of tadpoles of *Rana pipiens* 18 days after hatching showed a very high activity of tyrosinase per microgram of DNA. Thereafter the activity decreases to 30 to 40% of the 18-day level and remains so for 150 days. Lenses of adult *Rana pipiens* show no tyrosinase activity at all when assayed with the same method.

When the activity is based on protein rather than on micrograms of DNA, a continual decline of tyrosinase activity with development is found. This decline reflects the increasing accumulation of lens proteins which is the prominent feature of lens development. When the activity per milligram of protein at 18 days is put at 100%, the value declines to 15% at 30 days, 4% at 60 days, and 1% at 150 days. The data suggest that a high tyrosinase activity is common to the growing lens whether it is formed in normal development or by a regenerative process.

3.47 IN VITRO TRANSFORMATION OF LENS EPITHELIUM INTO FIBERS AND CONTROL OF GAMMA CRYSTALLINS

Tuneo Yamada D. S. McDevitt[1]

In the growing vertebrate lens, the cells of lens epithelium are gradually transformed into lens fibers at the lens equator. Some experimental data suggest that this transformation is controlled by a factor present in the posterior optic chamber. To elucidate the mechanism involved in this cellular transformation, larval lens epithelium of *Rana pipiens* was cultured in vitro. A fragment of lens epithelium together with lens capsule was separated from the lens, wrapped in a piece of hen's vitellin membrane, and put in hanging drop culture. L-15 adjusted to amphibian cells with or without 10% calf serum was used as medium. Histological observations were made on serial sections of 5-μ thickness after Bouin fixation, and immunofluorescence observations were made on 3-μ-thick sections after cold 95% ethanol fixation. The antibodies against *Rana pipiens* total lens proteins and those against the purified *Rana pipiens* gamma crystallin fraction were used for indirect immunofluorescence tests. Specificities of those antibodies were tested and found satisfactory.

Histological observations demonstrated that the lens epithelium can be cultured in L-15 with serum at least for eight days at 22°C. During the culture the cuboidal cells become gradually elongated and increase their cytoplasmic mass. Those cells indicate strong mutual affinity and produce a compact mass of fiber cells, often covered by a layer of capsule. Some of the explanted cells become pycnotic, separate from the fiber mass, and disintegrate. The lens epithelium before the culture is positive for the total lens protein test but negative for the gamma crystallin test. The cultured tissue becomes gradually positive for the gamma crystallin test as well. In the seven-day series, all explants tested were positive for the gamma crystallin test. In contrast to the culture medium with calf serum, the medium without calf serum failed to support transformation of lens cells. The test for gamma crystallins remained negative in all explants cultured in the latter medium. The results indicate that control of gamma crystallin synthesis in the lens cells in vitro is closely comparable with that in vivo, which was studied earlier in our laboratory. Gamma crystallin synthesis is closely associated with lens fiber differentiation in both systems.

Reference

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3.48 KILLING OF *HAEMOPHILUS INFLUENZAE* CELLS BY INTEGRATED ULTRAVIOLET-INDUCED LESIONS FROM TRANSFORMING DNA

K. L. Beattie Jane K. Setlow

Highly competent cultures of *Haemophilus influenzae* are inactivated by exposure to transforming DNA irradiated with ultraviolet light (uv). As a function of uv dose to the DNA, the killing goes to a maximum and then decreases. Killing is greater in a strain unable to excise pyrimidine dimers. The previously reported killing of *H. influenzae* by unirradiated *H. parainfluenzae* DNA is enhanced by low doses of uv, but drops off at high doses. Since there are no such lethal effects in a strain of *H. influenzae* that takes up DNA normally but does not integrate it, it is concluded that the killing is associated with integrated uv lesions.

In the case of wild-type cells, the lethal lesions from transforming DNA are pyrimidine dimers, since all the killing due to irradiated DNA is eliminated by treatment of the DNA with photoreactivating enzyme from yeast which monomerizes all the dimers. However, maximally photoreactivated DNA does kill an excisionless strain of *H. influenzae*. Since the integration of irradiated DNA is the same in the two strains, these results indicate that a nondimer lesion is efficiently repaired in wild-type cells but not in the excisionless strain.

The decrease of killing observed at higher uv doses to the DNA may be explained only partially in terms of uv-induced loss of integration. For example, at a dose of 9000 ergs/mm^2, integration was reduced to 25% of normal, but killing was completely eliminated. It is postulated that the number of pyrimidine dimers relative to other DNA components integrated decreases

at higher uv doses. This hypothesis is in accord with the result that the nonphotoreactivable fraction of killing in the excisionless strain increases with increasing dose.

3.49 A NEW SELECTION METHOD FOR RECOMBINATION-DEFECTIVE *HAEMOPHILUS INFLUENZAE*

K. L. Beattie Jane K. Setlow M. E. Boling

In order to study the molecular basis of recombination in bacteria, it is important to obtain a number of mutants which are defective in this process, so that the biophysical and biochemical differences between those cells that can and cannot undergo recombination may be determined. We have developed a new selection process for such mutants of *Haemophilus influenzae,* based on the fact that the DNA of the related strain, *H. parainfluenzae,* kills around 60% of a competent population of *H. influenzae*, but only if such DNA can be physically integrated into the recipient genome. The method consists of repeatedly making cells competent and exposing them to the *H. parainfluenzae* DNA, followed by regrowth of the surviving cell population. After about 20 such cycles, a large fraction of the cells was immune to the lethal effect of the DNA, and showed the low level of transformation characteristic of recombination-defective cells.

The selection procedure was used on two different populations of wild-type cells: (1) a culture treated with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), and (2) a culture which was originally made highly competent and exposed to transforming DNA from *H. influenzae* strain DB117, a mutant isolated on the basis of its ultraviolet (uv) sensitivity which is defective in recombining both transforming DNA and phage DNA. We have screened more than 170 individual cell isolates from the MNNG series and more than 30 from the transformed series for transformation efficiency. Of these, 40 clones were selected for detailed study. Measurement was made of transforming DNA uptake into competent cells, uv and x-ray sensitivities of exponentially growing cells, and uv-induced delay in DNA synthesis. The results of this study may be summarized as follows: (1) The selection method is excellent for obtaining mutants which can take up DNA normally or almost normally, but are blocked in some further step in transformation. (2) The properties of strain DB117, which we earlier postulated to be a reflection of the same deficiency, namely, its inability to recombine DNA, its x-ray sensitivity, moderate uv sensitivity, and extraordinarily long uv-induced delay in DNA synthesis, are clèarly separable by transformation. For example, among 26 of the clones from the transformed series tested, none showed a DNA synthesis delay much greater than that of wild-type cells. Furthermore, the uv sensitivities were usually more like that of wild-type rather than like DB117. The x-ray sensitivities in both series ranged from wild-type to that of DB117, with many of the strains showing intermediate sensitivities.

It is concluded that the recombination mechanism in *H. influenzae* has little or no overlap with the uv repair mechanism, but there could be some overlap with the enzyme system involved in repair of ionizing radiation damage.

3.50 REPAIR OF ULTRAVIOLET-IRRADIATED TRANSFORMING DNA IN *HAEMOPHILUS INFLUENZAE*

K. L. Beattie Jane K. Setlow

Repair of uv-irradiated DNA has been inferred from the survival of cells, viruses, and transforming DNA after irradiation, because such survival is markedly lower in strains which are unable to excise uv-induced pyrimidine dimers from their DNA. Measurement of survival can only give the end result of maximum repair. The kinetics of repair of transforming DNA can be followed by exposure of the competent cells to irradiated transforming DNA, lysis of the cells at various times, and assay of the transforming activity of the lysates in cells which are themselves deficient in repair of uv-irradiated DNA.

Repair of transforming DNA in wild-type cells may be readily observed as an increase in activity from the irradiated donor DNA after its linkage to the recipient DNA. However, no repair has been observed in a mutant which is unable to integrate transforming DNA, although it takes up DNA normally and has a normal excision mechanism. Therefore we conclude that recombination of transforming DNA and the recipient DNA must take place before repair can occur.

Wild-type cells can repair nondimer damage in addition to dimer damage, as judged by the increase in transforming activity observed in lysates made with irradiated and maximally photoreactivated DNA. A small amount of repair was observed in one of the excisionless mutants, strain DB116, when lysates from this mutant were assayed on another, even more uv-sensitive, excisionless mutant. There is also a little repair of nondimer damage in strain DB116, although the rate of repair seems considerably slower than for

nondimer damage in wild-type cells. The repair in DB116 which operates in the absence of an excision mechanism may involve recombination between sister DNA strands, including part of the newly integrated material.

3.51 ULTRAVIOLET-INDUCED DECREASE IN INTEGRATION OF TRANSFORMING DNA IN *HAEMOPHILUS INFLUENZAE*

Amir Muhammed[1] Jane K. Setlow

Physical integration of transforming DNA is a necessary step in bacterial transformation. Ultraviolet (uv) irradiation of transforming DNA decreases its transforming ability. In order to understand the mechanism of this inactivation it is important to know how uv affects the various steps in transformation. We have therefore measured integration of transforming DNA as a function of uv dose, using a technique developed by Steinhart and Herriott. These workers have shown that competent cells containing radioactive DNA lose label to the medium upon exposure to transforming DNA in an amount equivalent to the amount of transforming DNA integrated into the cell's genome. Thus one can measure the amount of integration by observing the amount of label extruded into the medium from the competent labeled cell.

We have found that relatively large uv doses are necessary to decrease integration. Therefore the inactivation of transforming DNA is largely due to the incorporated uv lesions (mostly pyrimidine dimers) in the cell genome. The loss of integration is independent of the presence or absence of an excision mechanism in the recipient cell. Since we have previously shown that the excision mechanism contributes to the survival of irradiated transforming DNA, this result indicates that excision repair of pyrimidine dimer damage in transforming DNA takes place after integration.

The uv-induced loss of integration is largely eliminated by photoreactivation (whose biological effect is known to result from monomerization of pyrimidine dimers). Therefore dimers in transforming DNA inhibit integration, although some of them are also integrated. The $1/e$ dose for loss of integration produces pyrimidine dimers separated by about 400 nucleotides. This length of transforming DNA probably represents a piece of DNA which is just too short for a specific pairing between transforming DNA and cell DNA that is stable long enough for the recombination events to occur.

Reference

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3.52 INTERSPECIFIC TRANSFORMATION IN *HAEMOPHILUS*

K. L. Beattie Jane K. Setlow

Two aspects of interspecific bacterial transformation have remained unexplained for a long time. (1) The efficiency of transformation is always low in such crosses, although it has recently been shown that the amount of transforming DNA physically integrated may be almost as high as in normal homologous transformation. (2) The ratios of the efficiencies of transformation of different markers can vary considerably in interspecific as compared with normal transformation. It has been postulated that both of these phenomena may be explained in terms of excision of mismatched regions in newly incorporated transforming DNA. We have tested this hypothesis by investigating transformation by *Haemophilus parainfluenzae* DNA of various strains of *H. influenzae* which have or which lack an excision mechanism. The relative transformation efficiencies and the marker ratios are approximately the same in the two types of recipient cell. Furthermore, two markers which were closely linked in *H. influenzae* become unlinked in *H. parainfluenzae* DNA, regardless of the *H. influenzae* recipient. It is concluded that the low interspecific transformation and the changes in marker ratio cannot be explained on the basis of excision. The low transformation probably involves difficulties in expression of the markers in the mismatched DNA. The altered marker ratios probably reflect different degrees of DNA mismatch at different places on the genomes of the two species, resulting in varying amounts of pairing ability.

We have also investigated the relative ultraviolet (uv) sensitivity of transforming DNA assayed on a homologous or interspecific recipient. The uv-irradiated DNA appears *less* sensitive in the interspecific transformations whether the DNA comes from *H. influenzae* or from *H. parainfluenzae*. Dose-effect curves for the uv inactivation of transforming DNA normally appear on a semilogarithmic plot to have a steadily decreasing slope with increasing dose. Thus the more uv lesions (mostly pyrimidine dimers) in the DNA, the more difficult it is to inactivate it further. Our data on uv inactivation of DNA in interspecific transformation suggest that the

mismatched DNA regions behave like dimers in decreasing uv sensitivity.

3.53 THE RELATIVE EFFICIENCY OF REPAIR OF ULTRAVIOLET DAMAGE IN COMPETENT AND NONCOMPETENT *HAEMOPHILUS INFLUENZAE*

M. E. Boling Jane K. Setlow K. L. Beattie

Recently new methods have been developed for making highly competent cultures of *Haemophilus influenzae*, so that all but a small percentage of the cells are capable of taking up DNA. Therefore, it is possible to examine the radiobiological properties of competent cells, even without exposing them to transforming DNA.

We have found that competent wild-type cells show a greater sensitivity to uv inactivation than noncompetent cells. This phenomenon apparently involves the decreased ability of the competent cells to repair uv damage, since a uv-sensitive strain which lacks the ability to excise pyrimidine dimers from its DNA does not become any more sensitive in the competent stage. Since uv-irradiated *H. influenzae* phage DNA is repaired in some host cells, we might expect to find a similar difference in survival of such phage in competent and noncompetent cells. However, although there is some increase in phage sensitivity in competent cells, what is apparently changed is merely the fraction of cells which are able to repair the phage, since the final slope of the second part of the two-component survival curve is the same, although the extrapolation of this curve occurs at a lower survival level. In general, competence affects cell survival more than it does phage survival.

Since competence is believed to be mostly an alteration of the cell membrane, these results suggest that at least part of the repair of cellular DNA takes place at the membrane. However, the smaller effect of membrane alteration on repair of phage DNA indicates that such repair, although it involves host cell enzymes at least in part, is not so firmly localized. This is in accord with our inability to observe competition for phage repair by uv lesions in the cellular DNA.

3.54 DEPENDENCE OF RECOMBINATION OF *HAEMOPHILUS INFLUENZAE* BACTERIOPHAGE ON THE HOST CELL

M. E. Boling Jane K. Setlow

We have previously advanced the hypothesis that a transformation-defective and ultraviolet-sensitive strain of *H. influenzae*, DB117, is defective in recombining DNA. We have tested this hypothesis by measuring vegetative phage recombination in DB117 as well as in wild-type cells.

Four temperature-sensitive phage mutants were isolated following treatment of induced lysogenic cells with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. These phages, unlike the wild-type phage, cannot grow at 40°C but form plaques at 34°C. Wild-type or DB117 cells were mixedly infected with two different phage mutants at 34°C, and the progeny was examined for total phage and for recombinant phage able to grow at 40°C. The efficiency of the two strains of cells to produce progeny was the same at 34°C. However, whereas recombination could be measured in the wild-type cells at levels varying from about 0.003 to 0.008%, depending on which pair of mutants was used, none could be detected in DB117. A crude map was made of the mutations from the results of the crosses in wild-type cells. The two strains did not differ in their ability to be lysogenized by *H. influenzae* phage. However, lysogenized DB117 was not induced to produce phage, either by ultraviolet radiation or by mitomycin C, treatments which efficiently induce the wild-type lysogenic cell.

Three conclusions have been drawn from these results: (1) The *H. influenzae* phage uses the host cell recombination system for vegetative recombination and for excision of prophage, but not for lysogenization; (2) DB117 has a defective recombination mechanism for DNA in general; (3) phage recombination could be used as a screening system for recombination-defective mutants of *H. influenzae*.

3.55 ULTRAVIOLET PHAGE INDUCTION IN LYSOGENIC *HAEMOPHILUS INFLUENZAE*

Jane K. Setlow M. E. Boling

Ultraviolet irradiation of lysogenic *Haemophilus influenzae* cells causes production of phage, except in a *rec*$^-$ strain. Lower uv doses are required for induction in cells which are deficient in excision of pyrimidine dimers, and also in cells which are slow at sealing the single-strand gaps made by dimer excision. The spontaneous phage yield is always higher in cells which are made competent than in exponentially growing cells. The yield is again slightly increased by exposure of the competent cells to transforming DNA from nonlysogenic cells. Integration of the transforming DNA is required for this increase, since it does not occur in the *rec*$^-$ strain which takes up DNA normally but fails to

integrate it. A small amount of added induction results from the incorporation of dimers from transforming DNA into competent, lysogenic cells. This indirect induction is more readily detected in uv-sensitive cells than in wild-type cells, which are able to repair a considerable fraction of the incorporated dimer damage. It is concluded that production of phage may be caused by (1) recombination events in the genome as a whole and (2) dimers in the genome, either produced directly by uv irradiation or incorporated from transforming DNA. It is improbable that uv induction operates by stimulation of recombination, since (1) and (2) are additive.

3.56 THE ROLE OF RECOMBINATION IN TRANSFECTION OF *HAEMOPHILUS INFLUENZAE*

Jane K. Setlow M. E. Boling

Purified phage DNA can readily infect competent cells, a process called transfection. We have investigated the role of recombination in transfection by measuring it in wild-type and recombination-defective hosts, in the presence or absence of an excess of various other DNA's which enter the competent cell at the same time as the phage DNA. Transfection efficiency is always about an order of magnitude lower in the recombination-defective host, although the plating efficiency for whole phage is the same as for wild-type cells. Thus it appears that recombination is involved in infection by at least some of the phage DNA particles. The dependence of phage plaque formation on DNA concentration to some extent reflects the same phenomenon, in that for wild-type cells the number of plaques increases more rapidly with increasing DNA concentration than is the case for the recombination-defective mutant. These results suggest that some phage DNA particles do not contain sufficient information for phage replication, but these can recombine with other incomplete particles to form a complete genome.

Transfection in both types of host may be decreased several orders of magnitude by the addition of homologous (*H. influenzae*) DNA, but there is only a small decrease in transfection due to the presence of non-homologous (*Escherichia coli*) DNA, the latter probably resulting from competition for entering the cell. The competition by homologous DNA presumably requires pairing but not integration, since only homologous DNA can pair, but such DNA does not integrate into the recombination-defective genome although it competes somehow against phage DNA. It is concluded that phage DNA entering the cell must go to a certain site to begin replication, an event which may become improbable when homologous DNA is present and occupying the same site.

3.57 DEVELOPMENTAL MUTANTS IN *HABROBRACON*

R. H. Smith Margaret L. Yetté

Temperature-sensitive lethal mutations have been induced by chemical mutagens and radiation in *Habrobracon.*[1,2] These conditional mutations are expressed as lethals at 35°C and as wild-type (high viability) at 28°C. Conditional mutations, as do the regular or unconditional mutations, reflect genetic blocks in the normal developmental processes. However, because the temperature-sensitive ones can be maintained in the laboratory as stock cultures without special techniques, it is possible to carry out genetic and developmental studies on a large number of such mutants.

The time and pattern of gene action or gene product availability can be determined for each mutant by measuring adult survival after shifting the *Habrobracon* from 35 to 28°C and from 28 to 35°C at given time intervals during development from egg to adult. The time when no animals survive to adulthood after they are transferred from 35 to 28°C indicates that the gene product became necessary for survival, or that the gene has become active. The time when almost all animals survive to adulthood after they are transferred from 28 to 35°C indicates that the gene product is no longer necessary for survival, or that the gene has become inactive. The time span defined by this method is called the temperature-sensitive period.

Examples are given below to illustrate the temperature-sensitive periods of four temperature-sensitive lethal mutations induced with ethyl methanesulfonate.

tsl-6. In this mutant death occurs during the early to middle pupa stage when development is at 35°C. The temperature-sensitive period occurs just before the lethal phase, between 72 and 96 hr of development. The product made by this particular gene is necessary in this time period in order for the wasp to develop into an adult.

tsl-1. The temperature-sensitive period for this mutant is not as clear-cut as for *tsl-6*. The lethal phase is spread over the entire larval period and more or less coincides with the temperature-sensitive period, about 36 to 96 hr of development time.

tsl-5. The temperature-sensitive period for *tsl-5* suggests that the gene product is necessary for a length of time beyond the time and stage when death would occur normally when development took place at 35°C only. The lethal phase occurs sharply at the middle larval stage, while the temperature-sensitive period lasts from the late embryonic stage to the early pupal stage, or prior to the 24th hour of development until after the 96th hour of development.

tsl-2. This mutant is the most interesting we have characterized to date. Death occurs during the middle pupa stage. The gene product is necessary for survival only within the first 24 hr of development as determined by the temperature from 28 to 35°C. The shift from 35 to 28°C, however, suggests that the gene product is available, or can be made available, later in time to allow development to adulthood. The chance for survival declines the later the transfer is made.

We have isolated about 50 development mutants in *Habrobracon serinopae*. Temperature-sensitive periods have been obtained for eight of these mutants. So far, the developmental pattern (stage of death, temperature-sensitive period) is different for each mutant.

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[1] R. H. Smith, *Genetics* **60**, 227 (1968).
[2] R. H. Smith, *Mutation Res.* **7**, 231–34 (1969).

3.58 MUTATOR ACTIVITY OF RADIATION-SENSITIVE MUTANTS OF YEAST

R. C. von Borstel Katherine T. Cain
C. M. Steinberg

We have studied the effect of several radiation-sensitive mutants of yeast (*Saccharomyces cerevisiae*) on spontaneous mutation (Table 3.58.1). The mutational test system involves selection for lysine-independent reversions followed by testing to determine whether the reversion is at the *lysl-1* mutant locus or at a supersuppressor locus. For this system, locus reversions can be produced by a base-substitutional mechanism only, while suppressors can be produced by an addition-deletion mechanism as well.

Four x-radiation-sensitive mutants (*xrs*) showed rates of spontaneous mutation ranging from one to five times that of the control. In one case, base-substitution mutations only are enhanced, while in two others addition-deletion mutations only are enhanced.

Table 3.58.1. Spontaneous Mutation Rates of Strains of Yeast Which Differ in Radiation Sensitivity

Genotype	No. of Tests	Average Mutation Rate × 10^8		
		Locus	Supersuppressor	Total
Haploids				
+	5	0.5	4.2	4.7
xrs1-1	3	1.2	31.6	32.8
xrs2-1	1	1.9	3.8	5.7
xrs2-2	1	0.7	2.8	3.5
xrs3-1	1	0.5	12.1	12.6
uvs9-3	1	0.5	4.6	5.1
uvs9-2	1	0.4	1.9	2.3
uxs1-1	3	0.2	24.9	25.1
Diploids				
+/+	2	0.6	5.5	6.1
xrs1-1/+	1	0.4	3.1	3.5
xrs1-1/*xrs1-1*	1	0.9	26.5	27.4
uxs-1/+	1	0.6	6.1	6.7
uxs1-1/*uxs1-1*	1	0.6	19.4	20.0

Of the strains sensitive to ultraviolet radiation (*uvs*), one has the same mutation rate as that of the control, while another shows small (i.e., ½ times) antimutator activity.

We have only one strain sensitive to both types of radiation (*uxs*). The mutation rate of this strain in our system is six times that of the control. This enhancement is due solely to addition-deletion mutations.

Dominance studies have been carried out only with the two strains having the highest spontaneous mutation rates. In each case the mutants are recessive. This indicates that the mutator activity of these two strains is due to the loss of essential functions, perhaps that of enzymes which would ordinarily heal lesions that occur normally.

3.59 DOSE-INTENSITY RELATIONS FOR RECESSIVE LETHAL MUTATIONS INDUCED IN *HABROBRACON* SPERM

R. H. Smith R. C. von Borstel

Habrobracon sperm exhibits an exposure-intensity effect to x radiation when recessive lethal mutations are used as criteria. This effect is shown in Fig. 3.59.1. The acute radiation was delivered at an exposure rate of 120 r/min, and the exposures for the two exposure-response curves from protracted radiation were delivered over 42- and 65-hr periods. It is of interest to note that in each case the slope of the curve is 1.

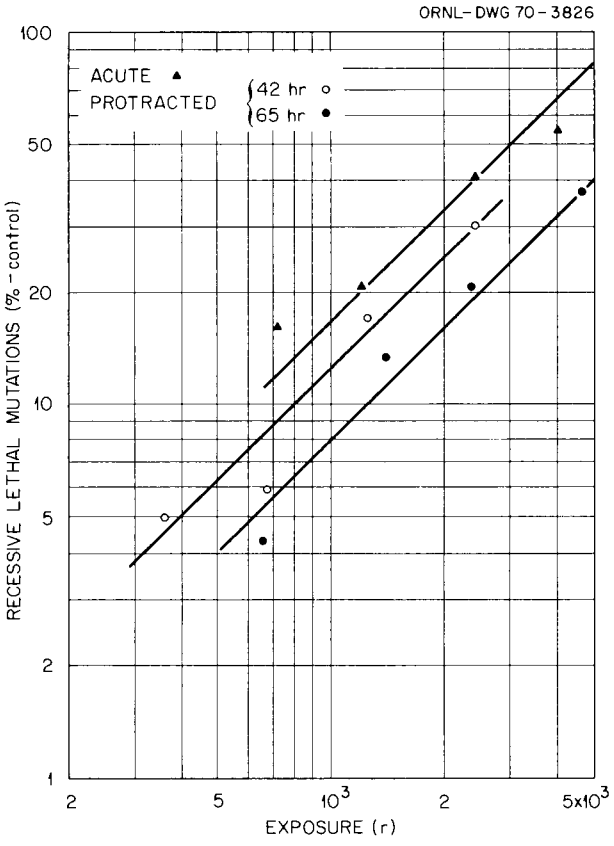

Fig. 3.59.1. Dose-Action Curves for Recessive Lethal Mutations in Sperm After Exposure to X Radiation and ^{85}Sr Radiation.

3.60 HYBRIDIZATION OF RIBOSOMAL RNA WITH SALIVARY GLAND CHROMOSOMES OF *RHYNCHOSCIARA*

R. C. von Borstel
Katherine T. Cain
Patricia F. Sanders[1]
Stella W. Perdue
C. Pavan[1]
F. J. Bollum[2]

Methods have been devised for demonstrating the molecular hybridization of ribosomal RNA with matching DNA in cytological preparations of nuclei.[3,4] These investigators labeled the ribosomal RNA with tritiated nucleosides and, after denaturing the tissue, detected the nuclear localization of the hybridized rRNA by autoradiographic means.

We elected to use a different approach to localize the regions of RNA hybridization with cytological preparations of chromosomes. Since it is possible to uniquely synthesize a new strand of DNA on a single-stranded DNA template with calf thymus DNA polymerase, then DNA which had been denatured in chromosomes can be demonstrated autoradiographically.[5] Therefore, if nonlabeled RNA or DNA would hybridize to denatured DNA in a specific region of the chromosome, this newly synthesized double-stranded molecule should arrest DNA polymerase action in this region. Preliminary studies showed that RNA preparations from yeast, *Chironomus*, or *Habrobracon*, when applied to the salivary gland chromosome preparations of *Chironomus* under hybridizing conditions, would quench the DNA polymerase reaction.[6]

Nonradioactive RNA was extracted from mature *Rhynchosciara* larvae by phenol, and the 28S fraction was isolated from the rest of the RNA on a sucrose gradient. We denatured the chromosomal DNA by several methods: 0.7 *N* NaOH, 0.01 *N* HCl at room temperature, heat, and 1 *N* HCl at 60°C. Results using the last method only are reported here. When *Rhynchosciara* salivary gland preparations had been treated with 1 *N* HCl at 60°C for 1 min, isotopically labeled 28S RNA can, under hybridizing conditions, bind to the chromosomes in specific regions.[7] This is shown in Fig. 3.60.1*c*. Several regions can be seen where the 28S RNA has bound to the chromosomes.

After the chromosomes had been denatured, the chromosomes were exposed to unlabeled 28S RNA under conditions where hybridization should occur. The preparations were then treated with calf thymus DNA polymerase and the four tritiated nucleoside triphosphate substrates. The nonhybridized control is shown in Fig. 3.60.1*b*. It can be seen that the labeling is uniform over the entire set of chromosomes. When the chromosomes had been hybridized to 28S RNA then several places showed a diminished labeling (Figure 3.60.1*a*). Particularly, these are the nucleolus-organizing region at the end of the X chromosome (lower right), the heterochromatic regions of chromosome A (central portion of the large chromosome in the upper part of Fig. 3.60.1*a*) and chromosome C (end of chromosome touching the X chromosome), and the nonheterochromatic end of chromosome B (chromosome at lower left in Fig. 3.60.1*a*).

The background is heavily labeled. Apparently the rather harsh denaturing treatment breaks strands of DNA from the chromosomes, and these stick to the glass of the slide or any exudate from the squashed preparations – the free DNA acts as templates for the DNA polymerase.

The method of hybridization using unlabeled RNA or DNA will be particularly useful in cases where it is difficult to obtain heavily labeled RNA or DNA

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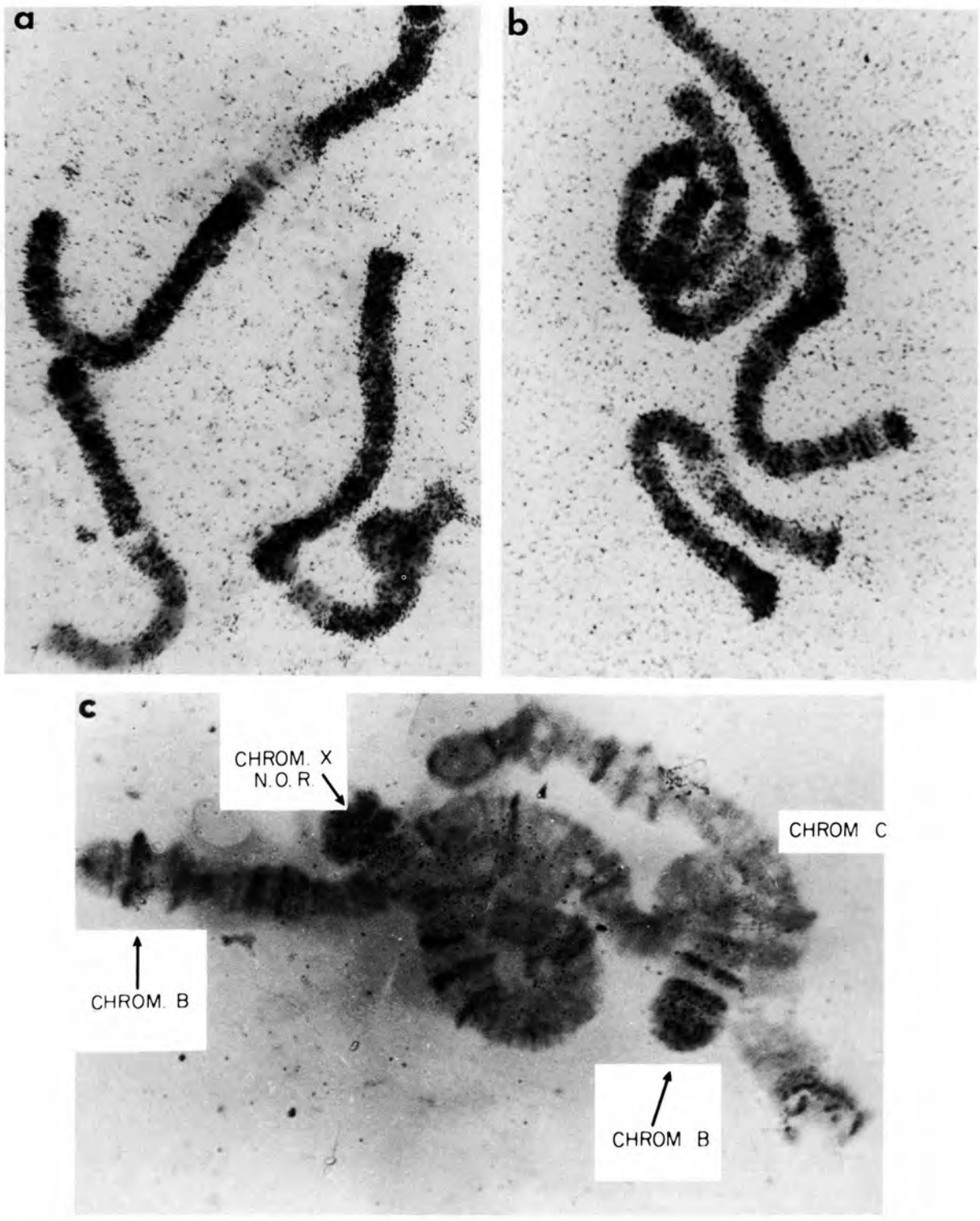

Fig. 3.60.1. Rhynchosciara Chromosomes. *a,* After denaturation, hybridization with 28S RNA, and treatment with DNA polymerase system. *b,* After denaturation and treatment with DNA polymerase system. *c,* After denaturation and hybridization with tritiated RNA. N.O.R. is the nucleolus-organizer region.

molecules for hybridization experiments. The usefulness of hybridization in cytological preparations is obvious from this experiment alone in that it showed that 28S RNA can hybridize at more than one place in the chromosome set.

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[7] A. L. P. Perondini, R. M. Macy, and C. Pavan, unpublished data.

3.61 THE RESPONSE OF *HABROBRACON* OOCYTES TO SPACE FLIGHT[1]

R. C. von Borstel — Margaret L. Yetté
Katherine T. Cain — Anna R. Whiting
R. H. Smith — Bobbie S. Brewen

One of the more puzzling findings in the Biosatellite-II experiment was the lethality induced in the *Habrobracon* oocytes which were in the first meiotic metaphase during the space flight. Though fecundity was higher for the metaphase I oocytes from nonirradiated females in the flight vehicle (380 eggs compared with 298 for those nonirradiated females in the ground-based control setup), the viability was distinctly lower (0.489 compared with 0.893). A similar circumstance was obtained for metaphase I oocytes from females that had been exposed to 2000 r of x radiation before the flight (fecundity: 147 and 94; viability: 0.565 and 0.93).

The stages at which the developing *Habrobracon* died are shown in Fig. 3.61.1. It can be seen that, with the exception of the earliest stage, the distribution of deaths throughout development is relatively uniform for the metaphase I oocytes from the flight animals that had not been irradiated. Death at stage I connotes dominant lethality, usually from induction of the breakage fusion bridge cycle. This can be seen in the metaphase I oocytes from the flight animals that had been irradiated before the flight.

An increase in frequency of deaths at stage 3 usually indicates death of the embryo from chromosome imbalance. This can be seen from the distribution of stages of death for offspring from females heterozygous

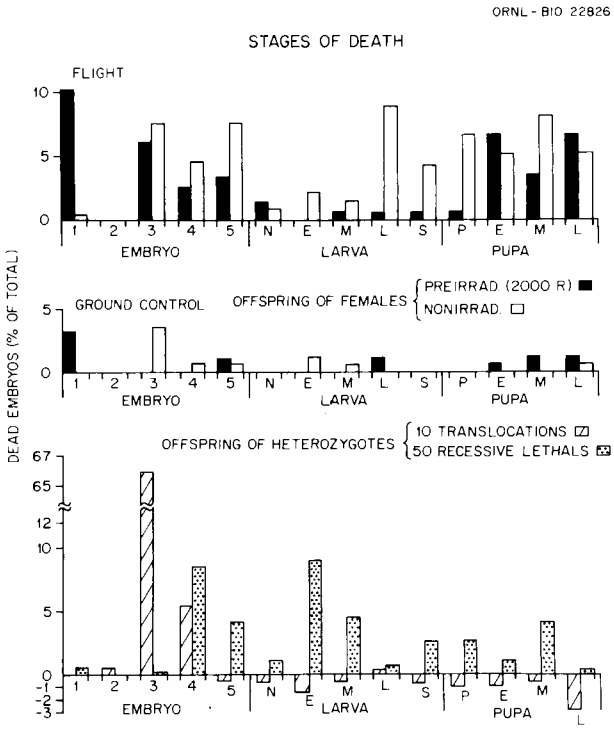

Figure 3.61.1. Stages of Death of Developing *Habrobracon* from Metaphase I Oocytes of Females That Were on the Biosatellite II Flight Vehicle and the Ground-Based Control Setup. The bottom histogram portrays the stages of death of developing *Habrobracon* from mothers that were heterozygous either for recessive lethal mutations or translocations.

for translocations (Fig. 3.61.1). The unweighted averages of times of death of offspring from 50 females heterozygous for recessive lethal mutations, shown on the same diagram, show that a fairly even distribution for times of death is obtained from about stage 4 throughout the rest of development.

Thus a case can be made that the excess of deaths found among the offspring of females that had been in the flight vehicle may be from a mixture of chromosome imbalance phenomena and recessive lethal mutations induced by the space flight conditions.

It was found that a preponderance of these dead offspring came from females carried in but one of the modules. We believe, therefore, that the low viability came from space flight stress other than weightlessness, or from weightlessness in combination with other factors of the flight.

Reference

[1] Research jointly sponsored by the National Aeronautics and Space Administration and the U.S. Atomic Energy Commission under contract with Union Carbide Corporation.

4. Mammalian Genetics Section

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4.1 EFFECT OF RADIATION DOSE RATE ON THE INDUCTION OF X-CHROMOSOME LOSS IN FEMALE MICE

W. L. Russell, Patricia R. Hunsicker, Elizabeth M. Kelly, Carolyn M. Vaughan, Linda K. Mann

The purpose of this experiment was to find out whether radiation dose rate, which has a marked effect on the frequency of induced mutations at specific loci, would have a similar effect on an entirely different type of genetic damage, namely, X-chromosome loss. The results would obviously be of basic interest as well as of immediate practical value in the estimation of genetic hazards.

Mature F_1 hybrid female mice from a cross of 101 and C3H strains were exposed either to 400 r of x rays, at approximately 80 r/min, or to 400 r of gamma radiation, at approximately 0.6 r/min, from a ^{137}Cs source. On the day following the exposure, the females were mated to males carrying the sex-linked gene Greasy (*Gs*). All the litters born in these experiments were conceived within the first seven weeks after irradiation. The offspring were scored for presumed *Gs*/0 females as well as for any other exceptional phenotypes. The presumed *Gs*/0 females were checked by breeding tests and chromosome counts. Chromosome counts of the mothers of these females were also made, to exclude any cases where the parent was already X/0.

In the x-ray experiment, there were from 50 to 64 *Gs*/0 females in a total of 7576 of these and *Gs*/+ offspring. The 64 include 14 cases in which the tests are not yet finished or in which the animals died before testing was completed. In the lower-dose-rate gamma-ray experiment the corresponding figures were from 21 to 27 in a total of 6674. The frequency in an unirradiated control group was from 3 to 4 in a total of 5547.

The frequency at the low dose rate is significantly below that at the high dose rate. The *P* value is 0.0007 (one-tailed test) when the maximum frequencies of 64 and 27 are compared. The difference is still significant ($P = 0.03$) even when the extremely conservative comparison is made using the minimum frequency of 50 in the high-dose-rate experiment and the maximum frequency of 27 in the low-dose-rate data.

The ratio of the induced frequencies of X-chromosome loss at low and high dose rates, again using the

maximum values, is 0.4. Thus there is a marked, statistically significant effect of radiation dose rate on this type of genetic damage in female mice. As with mutations detected by the specific-locus method, it would appear that the genetic hazard from a given total dose is less at a low dose rate than at a high one.

4.2 THE SEARCH FOR INVERSIONS ON SPECIFIC LINKAGE GROUPS OF THE MOUSE

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

In the search for inversions carried on at this laboratory, we have chosen to concentrate on several specific chromosomes in which, for one of several reasons, the availability of inversions would be of highest interest from the genetic point of view. To do this, we first screen genetically for crossover suppression in the appropriate linkage groups and then check suspicious cases cytologically. This procedure is in contrast to the inversion search carried out at the Jackson Laboratory, where the first screening is cytological, following which linkage mapping is attempted. Our calculations (based on frequency of nonrestituted breaks estimated from known translocation incidences) indicate that the probability of inducing an inversion in any one chromosome with the dose and germ-cell stage used is about 0.06%. Furthermore, inversions that do not include at least one of the markers would probably not be detectable by reduced recombination.

Experiments are in progress for four linkage groups: LG1, LG2, LG5, and LG20 (the X chromosome). In the recombination tests for the three autosomes, approximately 10,000 F_2 have been classified, testing about 200 irradiated chromosomes. Over 25,000 F_2 have been raised (only sons can be used for classification) from 536 tested X chromosomes. The X chromosome is of particular interest to us, since inversions in it would give critical evidence concerning our theories of the nature of X-chromosome inactivation. For this reason a disproportionate effort is being expended on the X, against great practical odds that exist both in the genetic and in the cytological testing.

In the inversion search on marked autosomes, cytological checks have been made in testis squashes. Approximately 900 anaphases have, to date, been analyzed in this manner from about 20 animals that yielded low-recombination progenies. Although two cases have shown 17% bridges each, no autosomal inversions have been clearly established to date. In the search for X inversions, it is unfortunately necessary to do the cytological work in hormone-ovulated females. In spite of careful elimination of all variables, only 0.9 analyzable anaphase can be obtained per female – due, largely, to the extremely short duration of this stage. From 22 females showing evidence of reduced recombination, 170 anaphases were examined in 188 progeny. Suggestive evidence has been obtained for the existence of two to three inversions. The most probable among these is a female that gave 3.9% recombination (instead of the 30% expected) and whose female progeny had 18.5% cells with bridges among 27 analyzed anaphases. Attempts are being made to maintain a line of this presumed inversion.

Work is currently in progress to improve the efficiency of both the cytological and genetic procedures. However, it should be remembered that, even under the best circumstances, the expected yield from this extensive experiment is low. As a by-product of the inversion search, we are finding a large number of translocations that also lead to reduced recombination and, frequently, to male sterility. Cytological analysis of the sterile males is proving to be of great interest.

4.3 CONTINUING STUDIES ON X-AUTOSOME TRANSLOCATIONS IN THE MOUSE

Liane B. Russell N. L. A. Cacheiro
Clyde S. Montgomery

X-autosome translocations in the mouse provide useful tools in the study of gene action, since the random inactivation which normally occurs in one X chromosome of a mammalian female is extended to autosomal loci, which normally act in double dose. Furthermore, the possibility exists that these rearrangements could provide cytological markers in the mouse karyotype, in which normally very few, if any, individual chromosomes can be identified. For these reasons a number of genetic, cytological, and phenotypic investigations on these translocations are being continued. A summary was presented last year[1] of extensive comparative studies in five T(X;1)'s with respect to various "vital statistics," the mapping of autosomal breakpoints, and the effect of the rearrangements on activity at four LG1 loci. This work confirms our hypothesis of an X-chromosome inactivation center or region and gradients of inactivation proceeding from this. The continuing studies have concerned themselves with determination of the X-chromosome breakpoints, cytological analysis of mitotic metaphases, and studies of the male sterility, which appears to be a constant property of T(X;A) heterozygotes.

Positions of X-chromosome breakpoints have been located relative to *Ta* and *spf* for four T(X;1)'s (R2, R3, R5, R6) and for one T(X;8) (R1). The results indicate

that, directionally, *c-p* on LG1 corresponds to *Ta-spf* on the X. R5, R1, and R6 are on the *Ta* side, outside the 30-unit-long *Ta-spf* segment, recombining 11, 6, and 6%, respectively, with *Ta;* R2 is about halfway between *Ta* and *spf;* and R3 is very close to *spf,* probably within the segment. The fact that both R3 and R6 give the fullest possible random inactivation of LG1 loci (i.e., a mean of 50% variegation), yet have their breakpoints in widely separated parts of the **X**, argues against Ohno's suggestion (1967) that X-chromosome material "as a unit" is required for random inactivation.

Karyotype analysis has been made of 188 mitotic metaphases from X-autosome translocation heterozygotes and of 20 from appropriate controls. In all five of the reciprocal T(X;1)'s and in both the T(X;8)'s, one translocated chromosome is clearly the longest in the complement, being 18 to 38% longer than the longest normal chromosome. In two of the T(X;1)'s, R5 and R4, the reciprocally translocated chromosome is identifiably shorter than the shortest normal autosome; in two others it may be approximately equal to the shortest; and in one it is probably longer. Calculations as well as karyotype analysis of the *flecked* insertion indicate that LG1 is probably no longer than 78% of the longest normal chromosome. LG8 is longer than LG1. The cytological measurements are in harmony with genetic determinations of breakpoints. Cytological studies of diakinesis are in progress.

All seven of our X-autosome translocations are male-sterile in extensive tests. Testis weights of 167 heterozygous translocation males were, on the average, only about one-fourth those of 125 nontranslocation segregants, although there are slight stock differences. Histological analysis by E. F. Oakberg indicates that no normal spermatogenic stages are found past pachytene in any of the rearrangements. Attempts at therapy have been made, and a number of combinations of hormones have been explored. One type of treatment has succeeded, in two of the stocks, to prolong normal spermatogenesis through diakinesis. This is of importance since the male meiosis can now be studied cytologically. So far, it has not yet been possible by modifications of the treatment to obtain normal postmeiotic stages. In another experiment, testis preparations from the various translocation stocks were stained with Acridine Orange. It was found that the heterozygous translocation males lack the bright orange spot (indicative of RNA) that is present in the sex vesicle of controls. (The sex vesicle, seen at pachytene, normally consists of the paired X and Y.) Since degenerative events may already be in progress, it is not clear whether the effect is a primary or secondary one.

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4.4 SEX-CHROMOSOME LOSS INDUCED IN MOUSE SPERMATOGONIA BY SINGLE AND FRACTIONATED DOSES OF X RAYS

Liane B. Russell Clyde S. Montgomery

Loss of any autosome is probably lethal in the mouse, while loss of a sex chromosome causes few adverse effects and is phenotypically detectable by use of appropriate markers. Induction of sex-chromosome loss has therefore been used by us in the past to compare a large number of germ-cell stages for radiation sensitivity to chromosomal damage.[1] Spermatogonia were among the few stages not previously studied, yet this cell stage is of the greatest importance in questions relating to human hazards of radiation.

A second purpose in studying induction of sex-chromosome losses in spermatogonia is to determine whether a certain type of dose fractionation, which has been found greatly to increase the frequency of specific-locus mutations,[2] will also augment chromosomal damage. [Another comparison between these two types of induced genetic damage is currently being carried out in females irradiated at different dose rates (see Sect. 4.1).]

(101 × C3H)F_1 males, 3½ months old, were irradiated with 600 r of x rays (250 kvp) at a dose rate of 66 r/min, either in one single dose or in two doses, 100 + 500 r, separated by 24 hr. Immediately after completion of irradiation, these two groups and a group of sham-irradiated controls were mated to *Gs/Gs* females for ten days in order to obtain spermatozoal data for comparison with our earlier work. Males were then removed and remated shortly prior to the estimated end of the sterile period and for the remainder of their life (spermatogonial data). Paternal sex-chromosome losses are detectable by the occurrence of *Gs*/0 daughters, which are tested genetically and cytologically.

Paternal sex-chromosome loss in controls is 0.14% to date (1 in 737) – well within the range earlier reported by us. Loss induced in spermatozoa was somewhat lower, but not significantly so, than our earlier reported frequency of 1.18% for 600 r. Offspring of irradiated spermatogonia are only now beginning to be produced in large numbers. To date, three cases of paternal sex-chromosome loss have been observed in 712 young. The present induced rate (irradiated minus control) is

therefore 0.28% for 600 r to spermatogonia. At this time the two irradiated groups do not differ significantly.

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[1] L. B. Russell, pp. 27–41 in *Effects of Radiation on Meiotic Systems,* IAEA, Vienna, 1968.

[2] W. L. Russell, *Japan. J. Genetics (Suppl.)* **40,** 128–40 (1965).

4.5 THE INSIGNIFICANT RATE OF INDUCTION OF SPECIFIC-LOCUS MUTATIONS BY FIVE ALKYLATING AGENTS THAT PRODUCE HIGH INCIDENCES OF DOMINANT LETHALITY[1]

W. L. Russell Sandra W. Huff Dorma J. Gottlieb

Five alkylating agents that had proved to be highly effective in inducing dominant lethal mutations in mice were tested for their capacity to induce specific-locus mutations. (Of the two which had also been tested for translocation induction, both were found to be effective mutagens for this effect too.) The compounds are: methyl, ethyl, *n*-propyl, and isopropyl methanesulfonates (MMS, EMS, PMS, and IMS respectively), and Myleran.

A single sublethal dose of the test compound was injected intraperitoneally into young adult F_1 hybrid male mice from a cross of 101 and C3H strains. The males were then mated to females of our T stock, which is homozygous for seven recessive marker genes. The offspring were examined for mutations at the seven genetically marked loci. The data reported here are restricted to matings occurring long enough after treatment to ensure that the male germ cells used in fertilization were all in the spermatogonial stage during the time of action of the chemicals.

A total of only five specific-locus mutations was obtained in 66,749 offspring. They were distributed as follows: MMS, 2 in 18,514 offspring; EMS, none in 14,787; PMS, 2 in 11,015; IMS, 1 in 9719; Myleran, none in 12,714.

The meager total of five mutations from all five compounds is not significantly higher ($P = 0.31$) than the spontaneous rate as determined in earlier experiments (28 mutations in 531,500 offspring). The mutation frequency for PMS, the highest in the group, is also not significantly above the spontaneous value ($P = 0.12$). Even arbitrarily selecting just those three compounds that happened to produce mutations (MMS, PMS, and IMS) and pooling the data from them still does not raise the mutation rate significantly above the spontaneous frequency ($P = 0.074$).

The ineffectiveness of these compounds in inducing specific-locus mutations is in marked contrast to their ability to produce dominant lethal mutations. The dominant lethal rates for these compounds at the chemical dose levels used correspond to those produced by more than 800 r of x rays for MMS, EMS, and PMS and to those from 600 r or more for IMS and Myleran. The number of specific-locus mutations induced by these compounds (if we assume that the slight, but insignificant, increase over the spontaneous frequency is in fact real) is less than one-fiftieth of the number that would have been expected from the above x-ray doses. If we make the extreme assumption that the present data have underestimated the specific-locus mutation rate to the extent that the true value is at the upper 95% confidence limit of the present figure, even this number of mutations would still be less than one-tenth of the frequency expected from the x-ray doses that match the dominant lethal effect.

This finding, of the relative ineffectiveness of these compounds in inducing specific-locus mutations, compared with their capacity for dominant lethal and translocation induction, is important and unexpected. It is in complete contradiction to published generalizations. For example, Auerbach[2] states: "Two major differences from X-rays were found early and seem to be characteristic of all alkylating agents, possibly of most chemical mutagens. One is a relative shortage of chromosome rearrangements compared with gene mutations. This is found for all alkylating agents. . . ."

Our data also show a major difference from x-ray results, but in exactly the opposite direction from that cited by Auerbach. We find a marked shortage, or even absence, of specific-locus mutations compared with dominant lethals and rearrangements.

It is clear that results in microorganisms, and even in *Drosophila,* can lead to highly misleading generalizations as to what will happen in mice.

Since the marked dominant lethal effects show that the chemicals are reaching the germ cells in concentrations sufficient for mutagenesis, one possible explanation for the shortage of specific-locus mutations may be that this type of damage is effectively repaired in the mouse. The evidence from radiation experiments that a repair system of this kind is much more effective in mice than in *Drosophila,* for example, suggests that differences in repair systems could easily account for species differences in pattern of response to chemical mutagens.

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[1] Research jointly sponsored by the National Institute of General Medical Sciences and the U.S. Atomic

Energy Commission under contract with Union Carbide Corporation.

[2]C. Auerbach, *Science* **158**, 1141 (1967).

4.6 RADIATION RESPONSE OF A_0 SPERMATOGONIA IN THE MOUSE

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

Recognition of the A_0 spermatogonia as the stem cells of the seminiferous epithelium makes them the most important cell type in both long-term fertility and genetic effects. The following experiment was designed to determine if differences in the surviving spermatogonial populations could be detected between irradiation doses which give different induced mutation frequencies per roentgen per locus.[1]

Hybrid 101 X C3H male mice were given injections of 12.5 μc of ^{3}H-thymidine at 5-hr intervals for a total of six injections. Twenty-four hours after the last injection, they were given single doses of 0, 100, 500, and 1000 r of x rays and the first 500 r of a fractionated 1000-r exposure; the second 500-r fraction was given 24 hr later. Mice were killed at intervals of 12 hr to 17 days after irradiation, autoradiographs prepared, and surviving spermatogonia scored.

Surviving spermatogonia were exclusively of the A_0 type after 500-, 1000-, and the fractionated 1000-r exposures. A few surviving A_1 cells were observed after 100 r. Difference in percentages of labeled cells was not apparent until 8.5 days after irradiation, or after one full cycle of the seminiferous epithelium (Table 4.6.1). A pattern then emerged which was maintained for a second cycle (until 17 days). In terms of mutation induction,[1] both 100 and 500 r should be on the linear portion of the curve, and they show an equal frequency (15.6 and 16.3%) of labeled cells at 8.5 days. The low percentage of labeled cells (2.4%) after 1000 r is associated with a lower mutation yield, and the highest frequency of labeled cells and mutations[1] (39.1%) occurs in the fractionated (500 + 500 r) group. The fact that the differences between groups are maintained until 17 days, with percentage of labeled cells in all groups one order of magnitude lower than the values at 8.5 days, is strong evidence that these differences are real. That this change by a factor of 10 also occurred in controls also indicates that normal kinetics of A_0 spermatogonia have been reestablished in the radiation groups and that these are the cells which will repopulate the testis.

These data are the first demonstration that differences occur among the spermatogonial populations which survive irradiation. It is also clear that a selective action of the second 500-r exposure is operating in the fractionated exposure. These differences may be a factor in the different mutation rates observed for these same doses, but a cause and effect relationship between these coincident events has not been proven.

Table 4.6.1. Percentage of Labeled Spermatogonia After X Rays

Time After Irradiation	Radiation Dose				
	0	100 r	500 r	1000 r	500 + 500 r[a]
24 hr	60.0	34.9	34.3	22.9	38.7
72 hr	15.9	47.4	57.7	55.7	59.3
5 days	13.4	46.7	62.9	59.0	59.8
8.5 days	7.8	15.6	16.3	2.4	39.1
17 days	0.7	1.6	1.7	0.2	3.1

[a]24-hr interval between 500-r dose fractions.

Finally, the data of Table 4.6.1, in conjunction with the concept that the A_0 is the continuously functioning stem cell and also is the most radiation-resistant spermatogonial type, indicate that all doses on the linear portion of the mutation response curve are based on the same cell population. Thus the highly sensitive cell stages are of transient nature, and extrapolation of the linear portion of the mutation rate curve should not underestimate the effect at low doses.

Reference

[1]W. L. Russell, in *Repair from Genetic Radiation Damage,* F. Sobels, ed., p. 205, Pergamon, New York, 1963.

4.7 SPERMATOGONIAL STEM CELL RENEWAL IN THE MOUSE

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

Historically, every cell of the seminiferous tubule, and even some outside the tubule, have been implicated as stem cells of the seminiferous epithelium. On the basis of recent ^{3}H-thymidine labeling experiments and a cytological study of type A spermatogonia, it has been possible to identify the A_0 spermatogonium as the stem cell and to delineate the general pattern of spermatogonial stem cell renewal.

Hybrid (101 X C3H) male mice were given injections of 12 μc of ^{3}H-thymidine at 5-hr intervals for a total of six injections (75 μc per mouse). Animals were killed at intervals of 1 hr to 17 days after the last injection, tissues were fixed in Orth's, and autoradiographs were prepared. A total of 1000 type A spermatogonia were

scored for each mouse. Three or more grains over the nucleus was considered positive for labeling. For cytological differentiation of the different types of A spermatogonia, tissues were fixed in Zenker-formol, sectioned at 5 μ, stained by the PAS reaction, and counterstained with hematoxylin.

Three morphological classes of type A spermatogonia could be identified, A_0 + early A_1, late A_1 + A_2, and A_3 + A_4. The A_0 spermatogonia are characterized by oval, heavily staining nuclei, and are present at all stages of the cycle of the seminiferous epithelium. This, plus the fact that they can be recognized as far as midprophase of mitosis, excludes the possibility that they are just cells in early interphase. The second category includes the late A_1 and A_2 spermatogonia, which have nuclei that are lighter staining, more rounded, and larger than those of the A_0's. One or two nucleoli usually are present. The late A_1 spermatogonia also occur throughout the cycle, but at markedly different frequencies. They are least numerous at stages I, II, and III, increase gradually to reach a maximum at stage IX, and disappear with formation of A_2 and A_3 spermatogonia at stages IX to XI. The final class, the A_3 and A_4, is identified by a nongranular, light-staining, somewhat rounded nucleus with small chromatin clumps on the nuclear membrane. This group of cells first appear at stage XI, reach a maximum at stage II, and completely disappear with the formation of intermediate spermatogonia.

Enumeration of the cell types throughout the cycle, with separation into the classes described above, suggests that the A_0 spermatogonium differentiates into the A_1 type, which, in succession, gives rise to the A_2, A_3, A_4, and intermediate spermatogonia. This concept of the A_0 spermatogonium as the active stem cell differs significantly from currently accepted models of stem cell renewal in which the A_0 spermatogonium is considered to function only in a "reserve" capacity.

The concept that the A_1 spermatogonia are formed by division of some A_4 cells at stage II of the cycle is at variance with our data, which indicate a gradual increase in A_1 spermatogonia at stages IV to IX. A_0 spermatogonia also can be seen in prophase at stages IV, V, and VI. Thus the population of cells which will initiate the multiplicative phase of their evolution toward formation of intermediate spermatogonia by dividing at stage IX develops over a period of time, and not from one particular point in the cycle.

Results from the labeling experiments also support the concept of the A_0 spermatogonium as the stem cell. Observations at 1 to 3 days are compatible with the model of stem-cell renewal proposed by Clermont and Bustos-Obregon,[1] but labeling at 5 to 17 days differs from what would be expected on the basis of the model. For example, some cells labeling at late VIII or IX appear as labeled A_2 and A_3 spermatogonia and some cells labeling at stages XI and XII appear as labeled A_4 spermatogonia $9\frac{1}{2}$ days later. If the model of Clermont and Bustos-Obregon[1] were correct, these cells should have divided four times and should not have been labeled. (Mean grain count initially was 11.5, with rare cells having 25 to 30 grains.)

It also was clear, both from morphological and labeling data, that the A_0 spermatogonia divide at all stages of the cycle. It is of particular significance that two qualitatively different types of divisions occur at stages I to II, IX, and XI. Since they are fewer in number and divide in conjunction with the more numerous A_1–A_4, the divisions of A_0 have not been recognized.

The model proposed on the basis of these data is that the A_0 spermatogonia are the stem cells of the testis. By a series of divisions, they give rise to the A_1 spermatogonia, which normally are committed to multiply and differentiate further into A_2, A_3, and other more mature germ-cell stages. The data suggest that the point of differentiation in the production of new A_1 spermatogonia probably occurs one cycle of the seminiferous epithelium earlier than indicated in previous models of stem cell renewal.

Reference

[1] Y. Clermont and E. Bustos-Obregon, *Am. J. Anat.* 122, 237 (1968).

4.8 CHEMICALLY INDUCED DOMINANT LETHAL MUTATIONS AND CELL KILLING IN MOUSE OOCYTES IN THE ADVANCED STAGES OF FOLLICULAR DEVELOPMENT[1]

W. M. Generoso Sandra W. Huff Sandra K. Stout

Eight alkylating compounds are extensively under study for genetic effects in mice: ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), *n*-propyl methanesulfonate (PMS), isopropyl methanesulfonate (IMS), 1,4-di(methanesulfonoxy)butane (Myleran), triethylenemelamine (TEM), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), and 2-methoxy-6-chloro-9-[3-(ethyl-2-chloroethyl)aminopropylamino]-acridine dihydrochloride (ICR-170). EMS, MMS, MNNG, and ICR-170 have already been tested for induction of dominant lethals in female mice.[2] The

present study involves (1) the extension of this genetic test to include IMS, PMS, Myleran, and TEM and (2) histological examination of ovaries after IMS treatment.

Female mice (11 to 13 weeks old) from T-stock, (101 X C3H)F_1, and (SEC X C57BL)F_1 strains were injected intraperitoneally with one of the following doses: 75 mg/kg of IMS, 600 or 500 mg/kg of PMS, 40 mg/kg of Myleran, or 1.6 mg/kg of TEM. These doses were adjusted for the average body weight. IMS, PMS, and TEM doses were prepared in Hanks' balanced salt solution (HBSS) and administered to each animal in 1-ml volumes. Myleran was suspended in olive oil and administered in 0.5-ml volumes. Control mice were injected with 1 ml of HBSS or 0.5 ml of olive oil. Experimental and control females were mated with (SEC X C57BL)F_1 males at the following posttreatment intervals: ½ to 4½, 5½ to 9½, 10½ to 14½, and 15½ to 19½ days. Females were opened for classification 12 to 15 days after observation of the vaginal plug.

Results shown in Table 4.8.1 revealed the high mutagenic effects of IMS and Myleran in the three strains. Clear effects of Myleran can be seen as reduced average numbers of living embryos in the four mating intervals and corresponding increases in the frequency of dead implantation. These effects were generally of the same degree in all four intervals, and there is little evidence for the existence of strain differences.

The strong mutagenic action of IMS, like that of Myleran, can be seen as reductions in the average numbers of living embryos and corresponding increases in the frequency of dead implantations in the three strains at all mating intervals. But, unlike Myleran, IMS caused drastic reductions in the frequency of fertile matings and in the average number of total implantations in T-stock and (SEC X C57BL)F_1 strains but not in (101 X C3H)F_1. Histological examination of ovaries revealed the basis for the large strain differences in these responses. In the three strains, IMS damaged the follicular cells of stage 6 to 8 follicles. However, while the oocytes in (101 X C3H)F_1 strain remained normal, in the other two strains the oocytes in the advanced stages of development were killed by IMS. All effects of IMS were higher in the earlier periods.

PMS induced low incidence of dominant lethals in T-stock female mice. Slight reductions in the average number 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 experiments involving the two hybrid strains was reduced to 500 mg/kg, and comparably treated T-stock females were maintained. This dose had no appreciable effect on the frequency of dead implants and average number of living embryos in the three strains. Slight reductions in the frequency of fertile matings were noted in the three strains, but they 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 PMS in a dose of 600 mg/kg. In the females of the two hybrid strains, TEM also induced a slight

Table 4.8.1. Induction of Dominant Lethals with Chemicals in Female Mice

Strain	Treatment	Dose (mg/kg)	Treatment-to-Fertilization Interval	Number of Females	Fertile Matings (%)	Average Number of Implants	Average Number of Live Embryos	Dead Implants (%)
T-stock	Control		½–4½	104	94.2	9.3	7.0	24.6
			5½–9½	71	94.4	8.6	7.0	18.9
			10½–14½	60	96.7	8.5	6.7	21.0
			15½–19½	62	96.8	9.3	7.4	20.5
(101 X C3H)F_1	Control		½–4½	82	89.0	7.2	6.7	6.8
			5½–9½	72	86.1	7.9	7.4	5.3
			10½–14½	68	94.1	8.2	7.8	5.0
			15½–19½	62	90.3	8.3	8.0	3.0
(SEC X C57BL)F_1	Control		½–4½	48	70.8	10.1	8.9	11.6
			5½–9½	47	74.5	9.8	9.3	5.0
			10½–14½	40	80.0	9.0	8.1	10.4
			15½–19½	41	78.1	10.4	9.7	6.9

Table 4.8.1 (continued)

Strain	Treatment	Dose (mg/kg)	Treatment-to-Fertilization Interval	Number of Females	Fertile Matings (%)	Average Number of Implants	Average Number of Live Embryos	Dead Implants (%)
T-stock	Myleran	40	½–4½	35	88.6	11.2	4.9	55.8
			5½–9½	33	72.7	9.2	5.4	41.2
			10½–14½	33	78.8	9.5	4.7	51.2
			15½–19½	30	80.0	9.4	5.4	42.5
(101 X C3H)F_1	Myleran	40	½–4½	44	59.1	6.7	2.9	57.1
			5½–9½	45	82.2	8.5	4.7	44.3
			10½–14½	44	86.4	7.3	5.1	30.8
			15½–19½	36	86.11	8.2	4.9	40.4
(SEC X C57BL)F_1	Myleran	40	½–4½	41	65.9	12.0	5.5	54.0
			5½–9½	42	71.4	10.2	4.3	58.2
			10½–14½	37	62.2	9.2	4.2	54.7
			15½–19½	42	88.1	9.9	6.8	31.6
T-stock	IMS	75	½–4½	36	8.3	4.3	0.3	92.3
			5½–9½	36	8.3	4.0	1.0	75.0
			10½–14½	29	17.2	7.2	1.4	80.6
			15½–19½	34	82.4	6.1	3.3	45.3
(101 X C3H)F_1	IMS	75	½–4½	44	77.3	6.1	3.1	48.5
			5½–9½	48	72.9	7.7	5.3	30.7
			10½–14½	41	85.4	8.5	6.3	26.3
			15½–19½	42	83.3	8.8	7.1	19.5
(SEC X C57BL)F_1	IMS	75	½–4½	42	19.1	4.6	0.4	91.9
			5½–9½	42	28.6	6.0	1.7	72.2
			10½–14½	42	40.5	5.8	2.9	49.0
			15½–19½	42	54.8	7.3	4.0	45.5
T-stock	PMS	600	½–4½	36	77.8	7.1	4.5	37.5
			5½–9½	31	83.9	7.5	5.1	32.0
			10½–14½	27	92.6	9.1	6.9	24.6
			15½–19½	33	90.9	8.4	6.4	23.7
	PMS	500	½–4½	35	65.7	8.9	6.4	27.5
(101 X C3H)F_1	PMS	500	½–4½	38	63.2	7.9	7.5	5.8
(SEC X C57BL)F_1	PMS	500	½–4½	41	58.5	10.1	9.6	4.9
T-stock	TEM	1.6	½–4½	26	84.6	7.5	4.9	34.1
			5½–9½	28	89.3	7.4	5.4	27.0
			10½–14½	30	93.3	9.2	7.5	19.0
			15½–19½	26	92.3	9.7	8.3	14.6
(101 X C3H)F_1	TEM	1.6	½–4½	40	85.0	7.4	5.9	20.6
(SEC X C57BL)F_1	TEM	1.6	½–4½	41	68.3	10.1	7.7	23.4

increase in the frequency of dominant lethals as shown by lower average number of living embryos and higher frequency of dead implants.

Of the eight compounds tested in three strains, all except ICR-170 induced dominant lethal mutations, but only IMS induced killing of oocytes in the advanced stages of follicular development in addition to this genetic effect. Large strain differences exist in both these responses. For example, one strain of females exhibited high sensitivity to dominant-lethal induction with EMS[3] and MMS while the other two strains did not, whereas in the case of IMS, dominant lethal mutations were induced in all three strains. On the other hand, the effect of IMS on oocyte killing was found only in two of the strains. Continuing studies now in progress involve the dominant-lethal response of the three strains to a lower dose of IMS and the histological examination of ovaries to study the toxic effects of EMS, MMS, PMS, Myleran, TEM, and MNNG on large follicles.

References

[1] Research jointly sponsored by the National Institute of General Medical Sciences and the U.S. Atomic Energy Commission under contract with Union Carbide Corporation.

[2] W. M. Generoso, *Genetics* **61**, 461–70 (1969).

[3] W. M. Generoso and W. L. Russell, *Mutation Res.* **8**, 589–98 (1969).

4.9 EFFECTS OF ALKYLATING AGENTS ON REPRODUCTIVE CAPACITY OF FEMALE MICE[1]

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Sandra W. Huff

The procedure used in the induction of dominant lethal mutations in female mice, described in Sect. 4.8, samples oocytes ovulated within 19½ days after chemical treatment. These oocytes are already in the advanced stages of follicular development. Although the length of time involved in the maturation of earliest oocytes into Graafian follicles is not yet known, it is already obvious that it is longer than 19½ days. The information presented here is on chemical effects on total reproductive capacity – an initial study on the effects of alkylating chemicals on mouse oocytes treated at different stages of follicular development.

(SEC × C57BL)F_1 female mice (11 to 13 weeks old) were given a single sublethal dose of one of the following compounds (see previous abstract for definitions) administered intraperitoneally: EMS (325 mg/kg), MMS (150 mg/kg), MNNG (70 mg/kg), ICR-170 (4 mg/kg), IMS (75 mg/kg), PMS (600 mg/kg), Myleran (40 mg/kg), and TEM (1.6 mg/kg). Control females received 1 ml of HBSS or 0.5 ml of olive oil. All females were caged permanently with (101 × C3H)F_1 males 24 hr after injection. Breeding pens were checked for presence of newly born babies, which were discarded after they were scored. This procedure in the mouse maximizes the number of young born to each mother. Data were pooled in the following intervals after mating (days): 18–24, 25–43, 44–62, 63–81, etc.

EMS, MMS, PMS, and MNNG had no effect on the average number of days between mating and birth of first litter and on the size of litters born in the early phase of reproductive life of treated females. Beginning with litters born in the interval 139 to 157 days after pairing, which roughly corresponds to the eighth litter, reductions in the average litter size were observed in these four compounds. MMS and PMS induced a slight but consistent reduction in the size of the subsequent litters. EMS and MNNG caused an almost identically pronounced reduction in the size of the subsequent litters. Reductions in the fertility of females in the MMS and PMS lots were not associated with reductions in the number of fertile females throughout the reproductive test period as compared with the controls. In the EMS and MNNG lots, reductions in the number of fertile females were observed later in the test period.

After IMS treatment only 3 out of 30 females delivered their first litters in the first interval. Most of the females (26 out of 30) produced young in the second interval, and the number of fertile females in the subsequent early intervals was normal. On the average the first litters appeared 11 days later than the controls (33.7 days after pairing compared with 22.7 days for controls). Litter sizes in the first four intervals were markedly lower than the controls. In the next four intervals, litter sizes increased considerably, although they were still slightly lower than control levels; and then in the subsequent litters, a steep decline similar to that with EMS and MNNG was observed. There was a significant reduction in the frequency of fertile females in the later intervals. This effect was similar to those of EMS and MNNG.

In the Myleran experiment the proportion of females that delivered their first litters during the first period is slightly lower than in the control (17 out of 29 compared with 24 out of 30 for the controls). Most of the females had litters in the second period (23 out of

29). On the average the first litters appeared six days later than controls. In the third period, most of the females stopped reproduction (only 7 out of 29 had babies), and in the subsequent periods even fewer females were fertile. Myleran-treated females produced an average of only 2.0 litters. The average sizes of litters produced at all intervals were markedly smaller than controls.

TEM had no effect on the proportion of females that had babies in the first two intervals. During these periods the average litter sizes were also normal. In the third interval, most of the females, like those treated with Myleran, stopped reproduction (only 7 out of 28 had babies). The average size of these third-interval litters and of the litters produced in the subsequent periods by even fewer females were smaller than controls. TEM-treated females produced an average of only 2.8 litters.

ICR-170 had no effect on the reproductive capacity. EMS, MMS, PMS, and MNNG had no effects on early litters but reduced the size of later litters. IMS affected early and late litters. Myleran drastically cut short the reproductive life of females, and size of litters produced was markedly lower than controls. TEM had no effect on early litters but markedly cut short the reproductive life. These results demonstrate very clearly a wide range of fertility effects of alkylating chemicals on (SEC X C57BL)F_1 female mice. The effects of lower doses of all the chemicals mentioned above, with the exception of ICR-170, and histological examination of ovaries to study fertility effects are in progress.

Reference

[1] Research jointly sponsored by the National Institute for General Medical Sciences and the U.S. Atomic Energy Commission under contract with Union Carbide Corporation.

4.10 METABOLIC FATE OF ETHYL METHANESULFONATE AND METHYL METHANESULFONATE IN THE MOUSE[1]

R. B. Cumming Marva F. Walton

Previous work in this laboratory and by others has shown that ethyl methanesulfonate (EMS) and methyl methanesulfonate (MMS) are very effective in producing dominant lethal mutations in both male and female mice and heritable translocations in male mice. Other types of genetic damage caused by these chemicals in mammals are still doubtful. The molar efficiency in the induction of dominant lethal mutations is about four times greater for MMS than for EMS. The present study was an attempt to find some basis for the large difference in biological effectiveness of these two compounds.

Methyl-^{14}C-MMS and ethyl-1-^{14}C-EMS were injected intraperitoneally with unlabeled MMS and EMS, respectively, to give a radioactive dose of 1 μc per gram of body weight and a total dose of 150 mg/kg of MMS or 400 mg/kg of EMS. The compounds were dissolved in Hanks' balanced salt solution and injected immediately after preparation. Eleven tissues were removed from animals at eight time periods ranging from 15 min to 24 hr after injection, and these tissues were stored in liquid nitrogen until assayed. The tissues were measured for total radioactivity, nonvolatile radioactivity, and relative activity in crude soluble, protein, and nucleic acid fractions. Autoradiograms were made of some tissue samples at various time periods. In other animals run simultaneously, excretion of $^{14}CO_2$ was continuously assayed until a respiration pattern analysis could be made, and urine was collected for determination of total activity in urinary metabolites.

Both MMS and EMS are rapidly distributed to all parts of the mammalian body after intraperitoneal injection. Total radioactivity in most tissues, including the testis, was about the same as the injected whole-body dose on a disintegrations per minute per milligram basis. Distribution was fairly uniform for EMS 15 min after injection and somewhat less so for MMS, with the greatest difference between the two being shown in the liver.

The amount of the parent compounds and their volatile metabolites decreased rapidly with time for both compounds, but the rate of decrease was greater for MMS. At 15 min after injection of EMS, 3.5% of the activity in the testis was nonvolatile (reacted to form metabolites which could not be dried out of the homogenate in a vacuum), while for the same time period and tissue with MMS, over 40% of the activity was nonvolatile. Soluble metabolites, proteins, and nucleic acids were also alkylated at early time periods to a much greater extent with MMS than with EMS.

An indication of the extent of hydrolysis of these esters to the corresponding alcohol and methanesulfonic acid is given by the pattern of excretion of labeled CO_2; $^{14}CO_2$ is excreted more rapidly and in larger quantities after administration of labeled EMS than after MMS injection. In the first 750 min after injection, a total of about 60% of the injected activity is recovered as $^{14}CO_2$ for EMS-injected mice. In those injected with labeled MMS, about 26% of the label is recovered in CO_2 in the same time period. These data

indicate that hydrolysis is an active, possibly enzymatic, process. No decision can presently be made on this matter for MMS.

In 24 hr after injection of labeled EMS, approximately 15% of the total injected activity is recovered as labeled urinary metabolites. The corresponding recovery after MMS injection is about 34%. The principal urinary metabolites for these compounds are derivatives of the S-alkylcysteines, though a number of minor metabolites occur. The recovery of these metabolites is an indication of the alkylating capacity of the agent in vivo, and again this capacity appears to be much greater for MMS than EMS.

There are large differences in metabolic patterns between EMS and MMS in the mouse, and these differences correlate with their unequal capacity to produce genetic damage. In general, EMS is hydrolyzed to a much greater extent, and MMS is much more efficient in alkylating all classes of biological molecules.

Reference

[1] Research jointly sponsored by the National Institute of General Medical Sciences and the U.S. Atomic Energy Commission under contract with Union Carbide Corporation.

4.11 CHEMICALLY INDUCED CHANGES IN THE EFFECTS OF CHEMICAL MUTAGENS IN THE MOUSE[1]

R. B. Cumming Marva F. Walton
William Winton

In an investigation on the influence of butylated hydroxytoluene (BHT) added to the diet of mice on the damage done by x rays and EMS, it was discovered that BHT protects against the effects of the alkylating agent but not against the effects of the radiation.

BHT was added to pelleted Purina lab chow at 0.75% w/w and fed to $(C3H_f \times 101)F_1$ or $(101 \times C3H)F_1$ mice from the age of 8 to 12 weeks. Control animals are from alternate boxes from production fed standard lab chow and kept on adjacent shelves in the same mouse rooms. When the mice reach the age of 12 weeks, they are all placed on regular lab chow, and the experiments are started.

In $(101 \times C3H)F_1$ male mice (48 in each group) given 725 r of whole-body x rays, the last BHT-prefed animals died on the 24th day following exposure, while 25% of the control mice survived more than 30 days. There was no apparent difference in the frequency of dominant lethal mutations induced in BHT-fed and non-BHT-fed animals following 600 r of partial body x rays. It is concluded if BHT confers any protection to mice against x-ray damage, it could not be measured by these tests.

When 40 each of BHT-fed and non-BHT-fed $(101 \times C3H)F_1$ male mice were given 525 mg/kg of EMS, all of the control animals died within two days, while 39 (97.5%) of the BHT-fed animals survived more than 30 days. Four additional experiments on BHT protection from EMS toxicity have been performed with somewhat different strain, sex, and dose relationships. These experiments involved 192 control mice and 187 BHT-prefed mice. All these experiments showed protective effects of the same magnitude as the one detailed above.

Two experiments were carried out to determine whether BHT would protect mice from EMS-induced dominant lethal mutations. The time period of these experiments was the first 20 days after EMS injection. In the first experiment the uterine contents of 304 female mice were examined, and in the second an additional 264 females were scored. Since the results of the two experiments were essentially the same, the data have been grouped. The dose of mutagen was 300 mg/kg of EMS. Control animals showed 100% dominant-lethal induction with preimplantation loss from the seventh to the ninth day after EMS injection. Corresponding BHT-fed animals reached a peak level of 50% dominant lethals on the seventh day after injection. In addition, we have shown in other experiments that BHT protects against EMS-induced heritable translocations in the mouse.

BHA (butylated hydroxyanisole) added to the food also protects male mice from EMS. BHT will protect female mice as well as males from EMS. Other data suggest that the basis of the BHT effect is the induction of drug-metabolizing enzymes by the chronic exposure to BHT. Therefore, other experiments have been carried out to find out how general the protection is with other alkylating agents and other potential mutagens.

A summary of preliminary results is as follows: BHT does not protect against methyl methanesulfonate (MMS) in males and confers only moderate protection in females. Isopropyl methanesulfonate, *n*-propyl methanesulfonate, and diethylnitrosamine are dramatically protected against with regard to toxicity by BHT in males, and moderate protection is conferred against ethylene dibromide. BHT does not protect against *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG).

Classic enzyme inducers such as sodium phenobarbital protect mice from the effects of EMS about as well as BHT does. Metabolic patterns using ^{14}C-labeled EMS

show that BHT protection is correlated with a higher hydrolysis rate of EMS in vivo and correspondingly a lower level of alkylation both in tissues and urinary metabolites.

Reference

[1] Research jointly sponsored by the National Institute of General Medical Sciences and the U.S. Atomic Energy Commission under contract with Union Carbide Corporation.

4.12 DIETHYLNITROSAMINE AS A POTENTIAL MUTAGEN IN THE MOUSE[1]

R. B. Cumming William Winton

Diethylnitrosamine (DEN) is a potent carcinogen in a variety of mammals and is particularly effective in causing lung adenomas, hepatomas, and squamous cell carcinomas in the mouse.[2] It is known to alkylate nucleic acids in vivo in the rat preferentially in the 7 position of guanine,[3] and it has also been reported to be mutagenic in *Drosophila.*[4] It has, however, been reported to be nonmutagenic in bacteria[5] and yeast.[6]

Most workers have suggested that the actual alkylating agent is produced metabolically in higher organisms by a process which involves enzymatic oxidative dealkylation of DEN. Malling[7] has reported that DEN becomes mutagenic in *Neurospora* when placed under conditions which would simulate this metabolic conversion.

We have shown that BHT (butylated hydroxytoluene) greatly stimulates metabolic processes involving some mutagens in the mouse. We therefore wished to check to see whether the toxic and mutagenic properties of DEN, which presumably require enzymatic activation, would be enhanced by BHT pretreatment. In view of the report that DEN ethylates DNA in vivo in a rodent, we wished also to look at its capacity to produce dominant lethal mutations in the mouse.

Toxicity of DEN. – Forty-eight (C3H × 101)F_1 male mice were fed BHT, 0.75%, in Purina lab chow from age 8 weeks to 12 weeks. Forty-eight control animals were handled identically in every way except that they received no BHT. Half of each group was injected with 150 mg/kg of DEN, and the other half of each group received 175 mg/kg of DEN. Of the 48 BHT-prefed mice, all survived in apparently good health for at least 30 days, and in fact there had been no mortality when the animals were discarded four months after injection. Of the control animals, 12 out of 24 receiving 150 mg/kg died within three days; the other 12 survived 30 days. All 24 of the controls which received 175 mg/kg died within three days.

Dominant Lethal Mutation Study. – A series of (C3H × 101)F_1 male mice which had been fed BHT (0.75% in Purina lab chow) for four weeks was divided into two groups, one of which received an intraperitoneal injection of 125 mg/kg of DEN and the other an injection of the same volume of Hanks' balanced salt solution (HBSS). In another series of non-BHT-fed animals, half were given DEN and half HBSS. The four groups of animals were then caged individually, each with three (C3H × C57BL10)F_1 females. The females were checked for plugs daily, and those which were mated were removed and replaced. Matings were continued for 30 days. Fifteen days after mating, each female was opened, and the uterine contents were scored.

The results of this test show that there is no indication that DEN induces dominant lethal mutations in any germ-cell stage, either when given alone or in combination with BHT pretreatment. There was in this test, as in past tests, a slight indication that BHT alone lowers fertility, but tests with a larger number of animals are needed to discover the basis of this effect. DEN, though it does not produce dominant lethal mutations, does prevent male mice from mating from the second until the ninth day after injection at this dose, and BHT pretreatment allows normal fertility of DEN-treated mice during this period. This antifertility effect is apparently a toxic and not a genetic consequence of DEN injection.

The fact that a compound which ethylates DNA in vivo does not produce dominant lethal mutations suggests that alkylation alone is not a sufficient condition for the production of this type of genetic damage.

References

[1] Research jointly sponsored by the National Institute of General Medical Sciences and the U.S. Atomic Energy Commission under contract with Union Carbide Corporation.

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[7] H. V. Malling, *Mutation Res.* **3**, 537–40 (1966).

4.13 BHT PROTECTION FROM EMS-INDUCED HERITABLE TRANSLOCATIONS IN THE MOUSE[1]

R. B. Cumming Marva F. Walton
Emily J. Turley

EMS (ethyl methanesulfonate) has been shown to be a powerful chromosome-breaking agent in the mouse. Not only is it very effective in producing dominant lethal mutations but also in inducing heritable translocations. It has been demonstrated in this laboratory that BHT (butylated hydroxytoluene), when fed to mice for four weeks prior to EMS injection, protects them from EMS-induced dominant lethal mutations and from toxicity. The present study was designed to determine whether BHT will also protect mice from EMS-induced translocations.

EMS (250 mg/kg) was injected into (101 X C3H)F_1 male mice which had been fed for four weeks prior to injection with a diet containing 0.75% BHT or into control animals which had been given an otherwise identical diet but without BHT. These males were then mated with T-stock females on the third day after injection, and plugged females were removed during the next four days. This procedure was followed so that offspring from these matings would be precisely timed and would have been conceived from sperm cells treated during the stage most sensitive to chromosome breakage by EMS. The F_1 offspring of these crosses were fertility tested using (C3H X C57Bl10)F_1 females or (101 X C3H)F_1 males as mates and classified as fertile, semisterile, or sterile.

Semisterility and sterility are indications of translocation heterozygosity, and these animals are being further tested to verify the translocations and to learn something about the types of translocations produced.

Of the control group we have tests complete on 93 F_1 males to date. Of these 52 are fertile, 24 are semisterile, and 17 are sterile. Thus 41 of 93 are apparently heterozygous for at least one translocation, or about 44%. Of the BHT-prefed group, we have tested 168 males to date, of which 135 are fertile, 20 are semisterile, and 13 are sterile. Thirty-three of 168 appear to be heterozygous for at least one translocation, or less than 20%. Tests are proceeding with other F_1 male offspring and with female offspring, but even at this stage it is apparent that there is a substantial difference between the two groups and that BHT does confer some protection against EMS-induced translocations in the mouse.

Reference

[1] Research jointly sponsored by the National Institute of General Medical Sciences and the U.S. Atomic Energy Commission under contract with Union Carbide Corporation.

4.14 TRANSLOCATIONS ARISING FROM EMS TREATMENT IN THE MOUSE[1]

R. B. Cumming N. L. A. Cacheiro

From an experiment in which we investigated the relationship of BHT pretreatment to EMS induction of translocations in the mouse (see Sect. 4.13), a number of translocations were obtained which are being studied more completely as a separate project. Some preliminary results are available from this study.

To date we have recovered, by fertility testing, 107 presumed translocations. When the testing program is complete, we should have about 200 EMS-induced translocations. Of the 107 presumed translocations recovered to date, 89 are in male mice and 18 are in females. This does not indicate that we expect fewer translocations in females but only that males can be tested more rapidly and more easily. Of the 89 translocations recovered from males, 31 (35%) are sterile, and the other 58 are semisterile. Cattanach *et al.*,[2] in an experiment involving smaller numbers of animals, concluded that about 50% of EMS-induced translocations recovered in males are sterile. We have recovered to date about four times as many translocations from males as Cattanach had, and we think that the final frequency of male sterile translocations will be closer to one-third than to one-half. Cattanach also reported that all of the translocations recovered from females were semisterile, with no sterility. He concluded that translocations which would produce sterility in males would cause only semisterility in females. We confirmed this for some of our translocations, but we have found two (11%) sterile females presumed to be translocations in this experiment to date. This indicates that EMS can induce chromosome rearrangements which will produce sterility in females as well as in males.

The animals mentioned above are under further study both from a cytological and a genetic point of view. To date we confirm Cattanach's conclusion that sterility or semisterility in offspring following the treatment of a parent with EMS is almost always due to a translocation. We have examined several of the semisterile males and found cytological evidence of translocation in meiosis of each of them. This cytological testing program continues. We have examined in detail 4 of the 31 sterile males recovered to date from this experiment. All four carried translocations. Three of the four had autosome–autosome translocations, and the other

shows a translocation between an autosome and the Y chromosome.

Translocations which can be carried as stocks will be so maintained and studied in an attempt to find whether there are qualitative differences between translocations recovered from EMS treatment and those induced by other means.

References

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4.15 THE OCCURRENCE AND SYNTHESIS OF GLYCERYL ETHERS IN CULTURED L-M CELLS

R. B. Cumming Fred Snyder[1]

Recent studies have shown that neoplastic tissues contain high levels of glyceryl ether diesters and that these compounds are not generally present in significant quantities in nonmalignant tissue.[2] Snyder and his colleagues[3] have recently described a microsomal enzyme complex from preputial gland tumors which is capable of synthesizing alkyl glyceryl ethers in a cell-free system from dihydroxyacetone phosphate and long-chain fatty alcohols. Essential cofactors for the formation of alkyl ether bonds in this system are ATP, CoA, and Mg^{2+}. In 1968 one of us (Cumming)[4] collaborated in the description of an in vivo–in vitro tumor system with L-M mouse fibroblasts which can be grown in a defined serum-free medium, in a medium containing serum, or as a tumor in a mouse. The cells are transferable from one type of culture to another and may be compared biologically and biochemically.

L-M cells in the chemically defined serum-free medium are rich sources of glyceryl ether diesters, these ethers composing about 20% of the neutral lipids of the cell (see ref. 5 for a fuller discussion). This is the highest percentage of glyceryl ethers reported for the neutral lipids of any mammalian cell. The L-M tumors contain approximately 8% glyceryl ethers in their neutral lipids, a value that is comparable with what has been found in other neoplasms. Thus this becomes an ideal system to study the relationship of glyceryl ether biosynthesis to the neoplastic state.

When microsomes are prepared from L-M cells grown in serum-free suspension cultures, these microsomes are very active in synthesizing glyceryl ethers in a cell-free system. Suitable substrates are long-chain fatty alcohols and either glyceraldehyde-3-phosphate or dihydroxyacetone phosphate, with the latter being a somewhat better glyceryl precursor at lower concentrations. Essential cofactors are the same as for preputial gland microsomes, that is, ATP, CoA, and Mg^{2+}. A preliminary report of this work has been accepted for publication,[6] and more-detailed studies are continuing.

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4.16 PLASMALOGEN BIOSYNTHESIS IN EHRLICH ASCITES CELLS GROWN IN ROLLER SUSPENSION CULTURES

R. B. Cumming R. Wood[1]

A roller bottle suspension culture system was used to study the biosynthesis of ether-linked lipids in Ehrlich ascites carcinoma cells by following incorporation of 1-^{14}C-1-^{3}H-hexadecanol into the various lipid classes as a function of time. Previous work with Ehrlich ascites cells in vivo[2] had shown that the alcohol serves as a precursor of alkyl glyceryl ethers in neutral lipids and phospholipids. Alkyl glyceryl ethers then undergo desaturation to the corresponding alk-1-enyl glyceryl ether. We were anxious to follow this process with Ehrlich ascites cells grown in culture to rule out possible metabolic interactions with the host mouse and to focus on the tumor cells alone. The cell culture method should also provide a cleaner system with better ability to follow the labeled precursor without competing irrelevant reactions in the host.

Cultures were set up in a roller bottle of our design using a modified 9-liter serum bottle as the culture vessel. Cells were transferred aseptically from the mouse using a syringe to approximately 1700 ml of medium 199 containing 10% calf serum in the roller bottle. Cells were maintained in suspension by rolling the bottle at approximately 6 rpm. Initial inocula were between 1.2

and 1.5×10^6 cells per milliliter of medium, and the cultures were maintained at 37°C. Under these conditions the cells grew well, with high viability during the first 48 hr.

Incubations were made at 6, 12, 24, 36, and 48 hr with 2.0 to 2.5×10^9 cells and 13 μc of 1-^{14}C-1-^{3}H-hexadecanol (specific activity: 39 μc/mg; ^{3}H/^{14}C ratio: 8.57). From 61 to 88% of the total administered radioactivity was recovered from the cell lipids, with the lowest figure being for the 6-hr incubation. Of the administered radioactivity, 77 to 93% was recovered from the lipids of the cells and media of the various experiments. This compares with a recovery of approximately 30% of the label administered in in vivo experiments reported previously.[2] The labeled alcohol is incorporated mainly into the ether-linked lipids, though some of it is oxidized to the acid. The lipid class incorporating the greatest amount of the label was phosphotidyl ethanolamine, though appreciable amounts were also incorporated into phosphotidyl choline and glyceryl ether diesters. Within these cells alkyl ethers incorporated label rapidly and then label in this group decreased with time, while alk-1-enyl ethers showed a corresponding increase in label. These results show that these cells synthesize plasmalogens by incorporating long-chain fatty alcohols into alkyl glyceryl ether linkages of phosphoglycerides and subsequently desaturating them to vinyl ether linkages.

The results show that the roller bottle system is an ideal technique for studies of this type.

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[1] Medical Division, Oak Ridge Associated Universities.

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Radiation Recovery of Hemopoietic Cells

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5.1 SECONDARY DISEASE IN RADIATION CHIMERAS[1]

C. C Congdon

The experimental-design–response-surface approach was used to investigate factors involved in 90-day secondary disease mortality of lethally irradiated mice treated with rat bone marrow. Secondary disease is a graft-vs-host syndrome that is associated with a mortality of about 65 to 95% in this transplant situation. When the factors age of donor cells, day of cell injection, dose of marrow cells, sex, and environment were examined for their main effects and interactions, a region of the factor space was found that gave about 25% 90-day mortality. The treatment conditions giving lowest mortality in the present experiments were: age of donor 3 to 33 days, day 1 injection of cells, a cell dose of 40×10^6 or higher, and an ultraclean environment of the unlimited filter-top caging type. Less than 10% of the mice surviving at 90 days had lost their rat marrow graft when tested for chimerism.

With situations giving low and high mortality from secondary disease in the same genetic situation, it should be possible to investigate the role that various pathological, biochemical, and immunologic alterations play in the pathogenesis of this graft-vs-host syndrome. When we can sort the significance of lymphatic tissue changes and the other processes it may be possible to circumvent secondary disease by taking advantage of the ability of many chimeras to cure the disease spontaneously.

Reference

[1] Work performed in collaboration with T. J. Mitchell and D. A. Gardiner, Mathematics Division, and M. A. Kastenbaum, Director's Division.

5.2 LYMPHATIC TISSUE CHANGES IN GUINEA PIGS EXPOSED TO CHRONIC GAMMA RADIATION

A. F. Van Pelt

This work involved a study of guinea pig tissues from experiments by Lorenz and Heston[1] carried out in 1941 and in the following years under the Manhattan Project. The animals had been exposed to several levels of external chronic gamma irradiation. All guinea pigs were from inbred strains 2 and 13. Some animals died in the radiation field, but others were allowed to live out their life-span after being removed from the field.[2] Nonirradiated control groups were also examined. In reviewing these slides, particular emphasis was placed on lymphatic tissue pathology, although all types of major and minor pathological conditions were identified and recorded. No significant differences were found in the numbers of germinal centers in lymphatic tissues between animals bearing tumors and those without tumors in the various experimental groups.

It was of special interest that guinea pigs exposed to the highest radiation level (8.8 per 8-hr day) showed marked destruction of bone marrow, but were able to maintain essentially "normal" numbers of germinal centers in spleen white pulp and lymph nodes.

References

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5.3 FURTHER STUDIES ON IMMUNOLOGIC MEMORY IN RADIATION CHIMERAS[1]

Nazareth Gengozian[2]

Previous studies demonstrated that allogeneic radiation chimeras in mice have an abnormal immune capacity as characterized by a reduced capacity to form antibody to particular antigens and a deficient memory system, that is, 7S antibody production. While the levels of 17S antibody formation in both a primary and secondary response to typhoid vaccine antigen compared favorably with those obtained in normal animals and syngeneic radiation chimeras, many allogeneic chimeras lacked the ability to form 7S antibodies. This immunologic defect was further characterized in additional allogeneic and xenogeneic (rat-mouse) chimeras with the soluble protein of *Salmonella typhi,* monomeric flagellin, and sheep and rat erythrocyte antigens. An attempt was also made to correlate the immune capacity of the various allogeneic chimeras with the severity of secondary disease as measured by body weight changes during the first 90 days after radiation and marrow treatment. Included in this group were animals that were originally part of a factorial design study aimed at reducing the incidence of secondary disease as measured by 60- and 90-day mortality.

Immune response studies were initiated 90 to 120 days post irradiation. All chimeras, allogeneic and xenogeneic, showed a reduced response to primary, secondary, and tertiary immunization with sheep and rat red blood cells; however, with these antigens the secondary and tertiary responses consisted of 7S antibody protein. With typhoid vaccine antigen and the flagellin protein, however, the memory response for 7S antibody was defective in most allogeneic chimeras. In any one particular strain combination of allogeneic chimeras, three types of responses were observed concerning this 7S memory system: (1) a complete absence, that is, no 7S production; (2) partial recovery with reduced 7S production; (3) a completely normal response. This defect in the recall response did not appear to be related to the severity of the secondary disease as gaged by body weight changes during the first 60 days post treatment; that is, even those animals which evidenced no sign of secondary disease by these criteria showed a reduced capacity to form 7S antibody. In those allogeneic chimeras where survival was greatly enhanced following consideration of several parameters related to inducing chimerism, the formation of 7S antibody in both the primary and secondary response was only slightly less than that observed in normal animals and syngeneic radiation chimeras. However, with variation in the dose of antigen and the time lapse between primary and secondary stimuli, the 7S memory system in these animals was still demonstrated to be defective.

References

[1] Work performed in collaboration with the Mammalian Recovery Group.

[2] Medical Division, Oak Ridge Associated Universities.

5.4 LIVER LIPIDS AND GRAFT-vs-HOST DISEASE

R. E. Toya

An alteration in the metabolism of liver lipids may be associated with graft-vs-host (GVH) disease. The GVH disease was induced by injection of 1×10^7 parent spleen cells (PSC) into 800 r x-irradiated hybrid mice. Total liver lipids were determined from the second through the seventh day after treatment by extraction in 2:1 chloroform:methanol. L-3-^{14}C-Serine was injected into treated mice, and serine metabolism was measured by respiration pattern analysis. The first death from GVH occurred seven days after treatment, with mortality reaching 100% on the ninth day. Total liver lipids were within control limits until the fifth day; on the sixth day, liver lipids were increased over controls by a factor of 2 or more. Thin-layer chromatography of total liver lipids six days after treatment with PSC revealed an increase in triglycerides in the neutral fat component and an almost total loss of phosphatidyl serine from the phospholipid component. Respiration pattern analysis of L-3-^{14}C-serine six days after treatment revealed an increase in the metabolism of serine. The almost total loss of phosphatidyl serine is the result of a lack of available serine for conversion to the corresponding phospholipid. The accumulation of lipid in the liver is concluded to be the direct result of altered serine metabolism induced by the GVH disease.

5.5 SERINE METABOLISM IN RADIATION CHIMERAS[1]

A. L. Kretchmar[2] E. J. Price[2]

In experiments utilizing experimental design techniques we found evidence of disturbed serine metabolism at two weeks after irradiation and treatment with bone marrow cells. This was before there was evidence of clinical graft-vs-host disease. The metabolic alteration is, therefore, not a trivial consequence of sickness but is a disturbance in metabolic pathways in animals which subsequently exhibit the morbidity and mortality of graft-vs-host disease.

The 100-day mortality was found to be greatest among those groups which showed highest incidence of altered serine metabolism at 14 days. In an accelerated form of graft-vs-host disease in mice treated with spleen cells, the disturbance in serine metabolism was detected as early as six days. In these mice, as in the mice given bone marrow cells, the respiration pattern of $^{14}CO_2$ after injection of L-3-^{14}C-serine was not affected by simultaneous injection of unlabeled formate. This suggests that there exists in graft-vs-host disease an alteration of the pathways of 1-carbon metabolism.

References

[1] Work performed in collaboration with the Mammalian Recovery Group.

[2] University of Tennessee Memorial Research Center.

5.6 SERUM ENZYME LEVELS IN ACUTE GRAFT-vs-HOST DISEASE[1]

W. R. Conover[2]

In graft-vs-host disease, necrosis may occur which results in metabolic alterations. These alterations, in turn, could be responsible for the decrease in liver

serine level which is characteristic of the disease. Since changes in levels of certain serum enzymes result from necrosis, we postulated that these enzyme levels may be abnormal in mice experiencing a graft-vs-host reaction. We studied, therefore, levels of several serum enzymes in F_1 mouse radiation chimeras injected with parent spleen cells.

$B6C3F_1$/MI female mice 12 to 16 weeks old were given 800 r total-body irradiation, and within 3 hr were injected with 10.0×10^6 spleen cells from C3H/Anf females 12 to 16 weeks old. On days 3, 4, 5, 6, and 7 following injection, blood was removed from the ocular sinus, and the serum was separated and, along with serum obtained from normal $B6C3F_1$ mice, analyzed for enzyme content using commercial reagent tubes and a Gilford model 2000 multiple sample absorbance recorder.

The enzymes studied were: lactate dehydrogenase (LDH), alpha-hydroxybutyrate dehydrogenase (HBD), glutamic pyruvic transaminase (GPT), isocitrate dehydrogenase (ICD), and glutamic oxalacetic transaminase (GOT), with special emphasis on LDH.

None of the enzymes studied showed significant variation from normal levels on days 3, 4, and 5 with the possible exception of GOT, which increased on day 5. HBD levels may be elevated on day 6, but extensive hemolysis makes the separation of clear serum difficult.

References

[1] Work performed in collaboration with the Mammalian Recovery Group.

[2] Loanee from University of Tennessee Memorial Research Center.

5.7 THE EFFECT OF LACTIC DEHYDROGENASE VIRUS ON LYMPHATIC TISSUES

M. R. Proffitt[1]

The lactic dehydrogenase virus (LDV) is known to be an unwanted contaminant in many transplantable tumors, as well as some pools of other animal viruses including the much-studied Rauscher leukemia vrius (RLV). Although LDV has shown no persuasive evidence of being oncogenic or even pathogenic, it does, nevertheless, induce a rather dramatic hyperplasia of lymphatic tissue germinal centers. These facts, coupled with evidence that germinal center cells may be targets for C-type viruses such as RLV, piqued our interest in LDV as a possible cocarcinogenic agent and led us to look more closely at its effect on the lymphatic tissues.

Eight-week-old specific-pathogen-free (SPF) male BALB/c mice were injected ip with 0.1 cc LDV having a titer of approximately 10^9 ID_{50}/ml. A similar group received an appropriate vehicle control injection. On days 1, 2, 4, 8, 16, 32, and 48, mice from each group were bled and autopsied.

Infection of mice with LDV caused a moderate elevation of spleen weight which persisted, although not at the maximal level, throughout the observation period. Accompanying this was considerable lymphatic tissue edema. Pyknosis and reduction of lymphocytes, as well as an early, dramatic, but transient, macrophage response in the "thymic-dependent" regions of the spleen and lymph nodes were also seen. During this early phase, there was a moderate, transient weight loss by the thymus which appeared to be reflected histologically in a reduction of small lymphocytes in the thymus cortex. Although there were considerable numbers of germinal-center-type cells present during the early phase of infection, typical germinal centers were not observed until after 16 days.

That similar observations have been made in animals infected with a pool of rapidly leukemogenic animal passage RLV known to contain LDV as a contaminant, but not in animals infected with LDV-free RLV propagated in cell culture and having a long leukemia latency, raises a question as to what role LDV might play in the overall Rauscher leukemia syndrome. The assumption can be made that LDV may behave as a cocarcinogenic agent. It may do this in at least three ways: (1) by increasing the supply of target cells for RLV, (2) by inducing an enhancing-type antibody, and (3) by interfering with tumor cell rejection through its effect on the thymic-dependent cells. It is this population of cells which is now thought to mediate the "allergic" cellular immune response.

Reference

[1] Predoctoral investigator.

5.8 RADIOPROTECTION WITH PHENYLHYDRAZINE

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

There is good evidence that a hypererythropoietic state renders mice more resistant to whole-body irradiation. For example, we have found that when phenylhydrazine – a drug which increases erythropoiesis – is injected into mice about a week before x irradiation, the $LD_{50/30}$ is increased 100 r or so. It is possible that an increase in hemopoietic stem cell number may be the

basis for increased resistance although other explanations are conceivable.

To evaluate the stem cell number hypothesis, the transplantation efficiency of (C3H × C57BL)F_1 bone marrow or spleen cells from phenylhydrazine-treated donors was determined in 900-r syngeneic recipients by the spleen-colony method or by scoring the number of 30-day survivors. Seven days after injection of phenylhydrazine, donor marrow and spleen cells were harvested and tested by both methods. No change in number or concentration of stem cells in bone marrow was observed. In spleen, however, there was a four- to fivefold increase in total cell number and a twofold increase in stem cell concentration, thereby increasing the hemopoietic stem cell number in spleen by eight- to tenfold. According to our estimates of total hemopoietic cell number in the mouse and of the marrow equivalence of the spleen, a tenfold increase in spleen cells represents approximately a 30% increase in total hemopoietic cell number per mouse. In terms of absolute numbers, marrow equivalence of the whole spleen would be around 200×10^6 cells on day 7 after phenylhydrazine injection. This is a sizable increase, and, according to the exponential survival curve for irradiated marrow cells, about 0.05×10^6 of the 200×10^6 cells would survive 800 r. Although these calculations are subject to error, they support the hypothesis that radioprotection afforded by phenylhydrazine results from an increase in hemopoietic stem cell number in the spleen. Furthermore, the presence of the spleen during irradiation is required for radioprotection by phenylhydrazine since splenectomy 30 days prior to drug injection abrogates the radioprotective effect.

5.9 EFFECTS OF SPLENECTOMY RELATED TO SURVIVAL AND BONE MARROW TRANSPLANTATION AFTER X IRRADIATION

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

The importance of the spleen to recovery of lethally x-irradiated mice injected with syngeneic bone marrow was evaluated. When splenectomy was performed before irradiation, injection of marrow resulted in 30-day survival which was the same as that for intact mice. However, when splenectomy was performed two or three days after exposure and marrow injection, survival was decreased, probably because the fraction of marrow committed to the spleen was removed.

Results of ferrokinetic studies (^{59}Fe) show that early regenerative erythropoiesis in lethally irradiated mice injected with marrow is reduced in animals splenectomized before irradiation but only when the marrow cell dose is limiting. Even at a limiting cell dose, erythropoiesis is eventually restored to that of intact mice. Thus the spleen appears to contribute to early restitution of a hemopoietic compartment least critical to survival of the animal, and whatever contribution the spleen makes to survival is compensated by other tissues when the spleen is absent.

Interpretation of splenic contribution to hemopoietic regeneration and survival is complicated by the fact that splenectomy performed two or three days before irradiation increases the $LD_{50/30}$ about 65 r. This kind of protective effect, however, is also manifested by uninephrectomy but not by partial hepatectomy or uniorchidectomy. Our data shed no light on the basis for this kind of radioprotection.

5.10 IS THE ROLE OF THE THYMUS IN HEMOPOIESIS DEPENDENT ON GRAFT-vs-HOST (GVH) REACTIONS?

Joan Wright Goodman Sarah G. Shinpock
Kay T. Burch

The ability of thymocytes to augment otherwise poor growth of parental (P) marrow in irradiated F_1 hybrids is restricted, with the exception of a small augmentation seen when F_1 thymocytes are given along with P marrow, to cells isogenic with the marrow (i.e., P). Inasmuch as P thymocytes are immunologically competent, there is a possibility that improved marrow growth results somehow from thymic GVH reactions. This possibility was explored in earlier experiments[1] in which lymph node lymphocytes were used instead of thymocytes, and in which marrow-donor:thymus-donor:host genetic relationships were so chosen that graft-vs-graft (GVG) reactions were either possible or impossible in the presence or absence of GVH. None of the results of these early studies indicated that GVH was essential to the thymic effect.

In the present study tolerant thymus donors, incapable of GVH, were used. They were radiation chimeras of at least three months standing, whose red cells were serotyped and shown to be of donor type at least two weeks in advance of their being used as donors. Cytotoxicity tests were carried out on pooled thymus cells before they were injected into irradiated recipients. Simonsen tests were also performed to analyze the thymocytes for tolerance. These tests showed that the thymus cells of the chimeras (of donor type) were specifically tolerant of the host but not of an unrelated F_1 hybrid.

Tolerant P thymocytes were as capable as nontolerant cells in augmenting erythropoiesis of transplanted P marrow in recipient mice. It can be inferred from these results that GVH is not a requirement for this particular thymic effect, although immunocompetence in general cannot be ruled out as necessary.

Reference

[1] Joan Wright Goodman and Sarah G. Shinpock, *Biol. Div. Ann. Progr. Rept. Dec. 31, 1968*, p. 118.

5.11 ROLE OF THE HOST'S SPLEEN IN IMPROVING MARROW GROWTH WHEN THYMOCYTES ARE GIVEN

Joan Wright Goodman Sarah G. Shinpock

A relationship between the thymus and erythropoiesis has been suggested by observations of several kinds over the years (see previous annual reports from this group). We have used a particular parent-to-irradiated-F_1-hybrid marrow transplantation system to study the relationship that exists in the mouse, because it is a system in which growth is poor but can be improved and easily measured.

Splenectomy was performed through a lateral incision in the abdominal wall under Metofane anesthesia. Blood vessels were cauterized with heat, and wound clips closed the skin incision. Sham splenectomy was performed by exteriorizing the spleen briefly and cauterizing a small amount of subsplenic tissue. Surgery was performed on ten-week-old mice. Two weeks later they were irradiated and given marrow with or without thymocytes.

Table 5.11.1 clearly shows that the spleen is essential to the effect: in the absence of the F_1 hybrid host's spleen, thymocytes were unable to produce the large augmentation of parental marrow growth usually seen. Parental thymocytes augmented marrow growth slightly in splenectomized hybrids, as indicated by erythrocyte ^{59}Fe uptake, but much less than in animals with spleens. In the absence of injected thymocytes, parental marrow grows even less well in splenectomized than in sham or unoperated hybrid mice. Although ^{59}Fe uptake by red cells was markedly greater in the presence of the spleen, ^{59}Fe uptake by femurs was slightly greater in splenectomized mice in both experiments.

The failure of thymocytes to potentiate hemopoiesis in the splenectomized mouse suggests that the spleen is the site of action of thymus-derived cells. Splenic ^{59}Fe-uptake data confirm that erythropoiesis was increased in that organ after administration of thymocytes. Viable thymocytes injected intravenously are known to localize principally in splenic white pulp and liver and to some extent in lymph nodes, but they are not found in appreciable numbers in the marrow. Even when the spleen was removed in the present experiments, thymus-derived cells apparently did not localize in the host's marrow, because erythropoiesis was not increased in the femurs of recipients of thymocytes. If reutilization of nuclear material is in any way involved in the potentiating effect of thymocytes, the material to be reutilized must be released in the immediate

Table 5.11.1. – Effect of Splenectomy of $BC3F_1$ Host on Marrow-Potentiating Effect of Parental Thymus

Pretreatment[a]	Postirradiation Treatment with Parental Cells		No. of Mice	^{59}Fe Uptake (% of injected dose; mean ± S.E.)		
	Thymus	Bone Marrow		RBC	Spleen	2 Femurs
			Experiment A			
Splenectomy	10^8	10^6	24	2.0 ± 0.2		0.7 ± 0.03
Sham splenectomy	10^8	10^6	18	6.2 ± 0.9	3.4 ± 0.2	0.5 ± 0.03
			Experiment B			
Splenectomy	10^8	10^6	26	1.3 ± 0.07		0.6 ± 0.02
Splenectomy	None	10^6	24	0.8 ± 0.06		0.6 ± 0.03
Sham splenectomy	10^8	10^6	23	6.0 ± 0.6	3.9 ± 0.3	0.5 ± 0.02
Sham splenectomy	None	10^6	21	1.8 ± 0.2	1.5 ± 0.2	0.5 ± 0.03
None	10^8	10^6	22	5.8 ± 0.9	3.7 ± 0.4	0.5 ± 0.02
None	None	10^6	28	1.8 ± 0.3	2.1 ± 0.3	0.5 ± 0.02
None	None	None	4	0.4 ± 0.1	0.3 ± 0.03	0.3 ± 0.04

[a] At ten weeks of age, two weeks before irradiation.

vicinity of the proliferating hemopoietic cell. Perhaps reutilization does not occur, but a stimulating substance is produced by living thymocytes. Again, our experiments indicate that the producing cell must be very near the target cell. There remains, of course, the possibility that neither a product of thymic cells nor reutilization of their nuclear material is required for the potentiation we are measuring. Instead, perhaps a cell-cell interaction requiring close contact of the two surfaces is necessary. That thymocytes were effective as a feeder layer in the experiments of Bradley and Metcalf[1] might be an argument against this possibility, because their hemopoietic cells growing in agar, although close to, were not in direct contact with feeder layer cells. It is not known for sure whether the effect we are measuring is related to what others have studied and described as syngeneic preference and allogeneic inhibition.[2]

References

[1] T. R. Bradley and D. Metcalf, *Australian J. Exptl. Biol. Med. Sci.* **44**, 287 (1966).

[2] K. E. Hellstrom and I. Hellstrom, "Syngeneic Preference and Allogeneic Inhibition," pp. 79–91 in *Isoantigens and Cell Interactions*, ed. by J. Palm, Wistar Institute, 1965.

5.12 LABORATORY ANIMAL TESTING PROGRAM

C. B. Richter R. W. Tennant J. A. Franklin F. S. Shults

Latent infection in laboratory animals remains a serious problem in radiation and carcinogenesis experiments, especially those involving mice. Where pseudomonas was previously a primary cause of aborted radiation experiments, other agents now appear to be involved, at least as cofactors in so-called "early death." For example, experiments employing combinations of chemical carcinogens and x rays have experienced problems with acute respiratory disease, possibly due to latent mycoplasma infections. Some mice used in bone marrow transplantation studies have apparently suffered fatal toxemias which relate to lack of environmental adaptation. Both of these problems involved previously unencountered experimental situations, making it necessary to investigate for corrective measures. Testing program observations on various hamster sources indicate that suitably "clean" animals of this species are not available. This could pose a relevant problem during long-term inhalation carcinogenesis projects because of nagging low-level mortality, apparently related to latent respiratory disease and poorly understood stress factors. Nevertheless, testing program information provided the necessary leverage in 1969 to obtain much "cleaner" rats for experimentation. This species has been notorious for its unsuitability for chronic studies because of spontaneous disease episodes. This same year repeated isolation of salmonella organisms from controlled areas indicated a primary feed contamination from the supplier, while a ruined experiment was traced to contaminated hatchery eggs as another source of salmonella.

The virus testing programs of the laboratory were consolidated, and the virus testing unit now processes specimens from all divisional programs. Intercurrent viral infections pose a particular problem in all carcinogenesis studies, and the purpose of the viral testing program is to aid in making available animals suitable for such studies. The testing program involves routine serodiagnosis using seven to nine different viral antigens, and in the last year these tests were applied to over 1400 animals. The program further has the capability to apply virus isolation and identification techniques to the solution of experimental problems.

In addition to approximately 50,000 regular accessions in the Testing Laboratory to guard valuable short- and long-term experiments, about 1000 specimens of special diagnostic interest to investigators were examined by the Testing Program. Most of the latter served to point out sources of error or helped correct experiment-related problems. In summary, the need for "reagent grade" animals will likely increase with increased emphasis on environmental studies. Such animals are not now readily available except in house. Plans for the present year emphasize directed effort in solving experiment-related problems such as early death.

5.13 AN APPROACH TO CLONAL ANALYSIS OF ANTIBODY-FORMING CELLS

J. F. Albright R. P. Quinn Alma L. Luzzati[1]

There are a variety of questions concerning the mechanisms of antibody synthesis and specificity that can best be answered through analysis of single clones of antibody-forming cells and the antibody that they produce. In principle such an analysis can now be accomplished. We present here a progress report of our

attempts to perfect and combine the various methods required for such an analysis.

In brief, the procedure is as follows. Irradiated recipient mice are injected with a small inoculum of syngeneic donor spleen cells designed to result in the deposition in the recipient's spleen of a single immunocompetent progenitor cell on the average. Sheep erythrocytes (Srbc) are employed as antigen. At a suitable time after donor cell injection, groups of recipients are sacrificed and their spleens removed. Each spleen is sectioned into three roughly equivalent pieces, and each piece is diced and cultured separately in vitro. The cultures are continued for 24 to 36 hr, and the antibody synthesized during this time is labeled by addition to the culture medium of ^{14}C-labeled amino acids. Upon termination of in vitro culture the spleen cells are recovered and assayed for hemolytic antibody-forming cells. Culture medium is collected from responding cultures and absorbed onto Srbc stroma. Subsequent elution of antibody from the stroma provides a preparation which can be analyzed by various immunochemical procedures designed to reveal: (1) specificity and homogeneity of the antibody, (2) molecular class of antibody, (3) quality of the component light and heavy chains of the antibody, and (4) presence or absence of key genetic markers on the antibody.

During in vitro primary response (initiated in vivo) there is an increase in the number of PFC. Antibody synthesis is linear for >36 hr and constitutes about half of the total protein synthesized. The rate of antibody synthesis is ~2400 molecules/PFC/sec. Over a 36-hr period sufficient antibody is released into the culture medium to provide detectable hemagglutination titers. There does not appear to be significant catabolism of released antibody during the 36-hr culture period. Immunoelectrophoresis of the culture medium followed by autoradiography permits identification of the quality of antibody produced.

Our immediate objectives are to determine whether or not (1) a given ancestral cell generates progeny that synthesize only a single class of immunoglobulin molecules and a single antibody specificity and (2) all the progeny of a given genetically heterozygous ancestral cell synthesize immunoglobulins coded for by one, but not both, allelic genes (the phenomenon of allelic exclusion).

Reference

[1] Visiting investigator from abroad.

5.14 A MODEL FOR THE INTERACTION OF CELL TYPES IN THE GENERATION OF HEMOLYTIC ANTIBODY-FORMING CELLS

D. L. Groves[1] W. E. Lever[2]
Takashi Makinodan

A series of limiting dilution and dose-response experiments involving the primary immune response of dispersed mouse spleen cells in Millipore diffusion chambers were completed as an extension of our earlier preliminary studies.[3] Based on critical analysis of the data, a stochastic model for the initiation of the immune response based on the interaction of two cell types is proposed. This model, which is more comprehensive than our earlier model,[3] suggests that an immunological response unit is formed by the multiplicative interaction of precursors of antibody-forming cells with antigen-reactive cells, the former cells becoming transformed into antibody-forming cells through such an interaction. That is, an individual antigen-reactive cell is able to interact with and influence the transformation of more than one precursor cell, and the number of precursor cells transformed per antigen-reactive cell is a function of the spleen cell dose. It has provided a satisfactory explanation for most, if not all, of the limiting dilution and dose-response data, including the more difficult dose-response data published recently. The ideas incorporated into this model clearly have implications for other areas of fundamental importance to immunology which will be discussed at a later date, for example, the problems of specific immunological tolerance and memory and the question of the precommitment of precursor cells to the synthesis of specific antibody molecules.

References

[1] Present address: Department of Microbiology, Wake Forest University, Winston-Salem, N.C.

[2] Mathematics Division.

[3] D. L. Groves, W. E. Lever, and T. Makinodan, *Nature* **222**, 95–97 (1969).

5.15 INVOLVEMENT OF A COMPETING ANTIGEN IN THE INTERACTION OF CELLS TO INITIATE ANTIBODY FORMATION

J. F. Albright T. F. Omer

At least two types of cells, the antigen-reactive cell (ARC) and the precursor cell (PC), must interact in

order for productive antibody-forming cells (AFC) to be generated. The ARC interact with antigen and are relatively radiation resistant; the PC give rise through mitosis to AFC and are radiation sensitive. Under appropriate conditions either the ARC or PC (or both) is preempted by a competing antigen such that the subsequent response to a given test antigen is markedly diminished. In this report we discuss evidence that suggests that it is the ARC which is preoccupied by a competing antigen.

Mice of the $C31F_1$ hybrid strain were injected with 2×10^9 sheep erythrocytes (Srbc) and three days later exposed to 850 r x irradiation. Shortly after irradiation the mice were injected with either (1) 12×10^6 normal spleen cells from syngeneic donors or (2) a mixture of 12×10^6 spleen cells and additional Srbc. At intervals of time thereafter groups of recipient mice were sacrificed, their spleens were removed and teased, and the dispersed cells were assayed for hemolytic antibody-forming (plaque-forming) cells (PFC). These experiments provided positive-control reference data. There was little difference in the number of PFC produced regardless of whether or not additional Srbc were inoculated along with the donor spleen cells. This result showed that the residual antigen present in the spleen subsequent to the initial injection of Srbc and radiation exposure was sufficient to trigger the generation of PFC by the PC in the spleen cell inoculum. It is reasonable to assume that the active antigen was that which was present in the ARC of the recipient or, at least, had been "processed" by those ARC.

Antigen-competition experiments were performed in a similar way. Prospective recipients were first injected with horse erythrocytes (Hrbc) and three days later exposed to x irradiation. They were then injected with a mixture of 12×10^6 donor spleen cells and 2×10^9 Srbc. The PFC response to Srbc was followed. Because the endogenous PFC in the spleens of recipient mice decayed rapidly after irradiation, it was possible to detect the formation of even a few ($\geqslant 16$) PFC by the inoculated spleen cells. The data clearly showed a marked reduction in the number of activated PC ($\leqslant 10\%$ of controls) and a marked prolongation of the latent phase preceding appearance of PFC.

From these experiments we conclude that it is the ARC that are influenced by competing antigens, and that they are not determined with respect to the antigen upon which they act. Whether or not a given ARC can "process" one antigen and then another, but not both simultaneously, remains to be determined.

5.16 DIGESTION OF ANTIGEN BY PERITONEAL MACROPHAGES

E. H. Perkins Charlene A. Seibert

Since Fishman's work in 1961, considerable evidence has accumulated in favor of the notion that peritoneal macrophages are essential for the initiation of antibody response. However, it is not generally appreciated that the spleen is the lymphoid organ almost solely responsible for antibody formation after a single intravenous or intraperitoneal injection of antigen. Therefore, antibody response in situ is dependent upon antigen reaching the spleen in an immunogenic form, and the response will be maximal only when the amount of antigen reaching this lymphoid organ is not limiting. We have previously demonstrated that peritoneal macrophages do not release or store immunogens after phagocytosis of sheep RBC, but destroy them and thereby reduce the effective antigen dose.

In the present investigations we have controlled the antibody response of mice by altering the number and functional activity of peritoneal macrophages. In addition to mice with normal peritoneal macrophage population, we used mice which had increased numbers of macrophages with enhanced functional activities. An 0.8-μ particle of polystyrene latex was used to block the uptake of sheep RBC by peritoneal macrophages. This was done by injecting intraperitoneally the latex particles 1 hr prior to antigen injection. Two times 10^7 ^{51}Cr-labeled sheep RBC were injected into mice by either the intravenous or the intraperitoneal route, and the localization of label in the spleen was assessed. The amount of antigen which reached the spleen was strikingly diminished when sheep RBC are injected intraperitoneally. This was particularly true in gelatin- and peptone-stimulated mice, which had increased numbers of active peritoneal macrophages. The injection of latex particles caused macrophages to engorge them, and the phagocytic capacity of the particle-engorged macrophage for sheep RBC injected subsequently was sharply curtailed. The antibody response, measured in terms of the number of four-day hemolytic plaque-forming cells in the spleen, was directly related to the amount of antigen reaching the spleen. Comparable results were obtained when ^{125}I-labeled aggregated human gamma globulin was used instead of sheep RBC.

It seems clear that the classical functional peritoneal macrophages cannot be considered as essential intermediaries for the initiation of antibody response.

However, other cells in the heterogenous population of cells harvested from the peritoneal cavity may be passively or actively involved.

5.17 RADIOSENSITIVITY OF IMMUNOLOGICALLY COMPETENT CELLS

G. B. Price[1]

Radiosensitivity of immunocompetent cells – in states of radiation-induced flux, continuing senescence, and steady-state equilibrium – is being studied. Flux was induced by 250-r x radiation. The recovery pattern of immunocompetence was fairly typical with a gradual recovery after reaching a minimal level three days after irradiation with an overshoot and return to normal levels subsequently. An abortive phase was observed six days post irradiation which had not been previously reported. It was found that the radiosensitivity of immunologically competent cells was not a function of the state of cells as previously reported;[2] D_{37} values were not significantly different when these cells were in exponential phase, abortive phase, or steady-state equilibrium. Preliminary evidence also indicates that the radiosensitivity of immunocompetent units in states of continuing senescence is unchanged.

References

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

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5.18 CONTINUING STUDIES ON THE IMPAIRMENT OF DIFFERENTIATION AND PROLIFERATION OF IMMUNOCOMPETENT AND SPLEEN COLONY-FORMING CELLS IN LEUKEMIC PRONE AKR MICE

E. H. Perkins Takashi Makinodan
A. C. Upton[1] Charlene A. Seibert
Lou C. Satterfield

Our recent studies demonstrated that when AKR/J(H-2^k) or C3Heb/FeJ(H-2^k) spleen cells were transferred with antigen (sheep RBC) into lethally irradiated young adult AKR/J recipients, the immune response in terms of peak numbers of direct and indirect plaque-forming cells was low. However, the immune response was high when the same number of spleen cells were transferred to lethally irradiated young adult C3Heb/FeJ recipients and intermediate when transferred to F_1 recipients. Comparable results were obtained for hemopoietic spleen colony-forming cells. It was concluded that the internal "milieu" or microenvironment of the spleen of the AKR mouse is defective for the support of the differentiation and proliferation of either antigen-stimulated immunocompetent or hemopoietic spleen colony-forming cells.

Further exploration of this deficiency has revealed that it is not confined to the splenic environment. Results comparable to the cell transfer studies were obtained by culturing antigen-stimulated spleen cells in cell-impermeable diffusion chambers which were implanted into the peritoneal cavity. This would implicate a humoral factor.

We were not able to relate the nature of the defect to histoincompatibility differences. Thus, assessment of recipient spleens for graft-vs-host (GVH) reaction revealed a strong GVH reaction when AKR marrow was transferred into lethally irradiated C3Heb/Fe recipients, a donor-recipient combination in which excellent growth of the lymphohemopoietic cells occurred. Only a questionable GVH reaction was observed when C3Heb/Fe marrow was injected into lethally irradiated AKR recipients, a donor-recipient combination in which growth of lymphohemopoietic cells was minimal. Additional studies involving transfer of C3Heb/Fe or AKR spleen cells with the antigen to other strains differing at the H-2 locus revealed that suboptimum response is not correlated with the known H-2 differences among donors and recipients.

As the thymus is invariably the initial target organ in the pathogenesis of this disease and thymectomy has been shown to drastically reduce the incidence of overt leukemia, cell transfer studies were carried out using thymectomized recipients. No difference in the number of plaque-forming or spleen colony-forming cells was seen between normal or thymectomized recipients. Injection of a significant number of C3Heb/Fe thymus cells together with the spleen cells and antigen into normal or thymectomized recipients was also without effect.

These results demonstrate that the x-irradiated leukemic-prone AKR does not support the growth of lymphohemopoietic cells. The cause appears to be the lack of an essential humoral factor or the release of a factor which suppresses differentiation and proliferation of lymphohemopoietic cells. In contrast, x irradiation of non-leukemic-prone strains of mice always supports maximum growth of lymphohemopoietic cells.

Reference

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5.19 THE PROTECTIVE CAPACITY OF "IMMUNOLOGICALLY PROGRAMMED" SPLEEN CELLS

E. H. Perkins Takashi Makinodan

As part of a study on immunologic rejuvenation of aged mice, the protective capacity of immunologically programmed spleen cells is being assessed. As a model system known numbers of "programmed" spleen cells from young adult mice previously immunized with *Salmonella typhimurium* are transferred to syngeneic recipients before and after storage at −196°C. The susceptibility of recipient mice is then determined by challenging them at varying intervals with a lethal dose of the virulent organisms.

Preliminary results revealed that programmed spleen cells can confer a significant degree of protection among young adult recipient mice. Thus 60% of young adult recipients challenged with 100 LD_{50} doses of *S. typhimurium* survived the infection when challenged 1 day to 14 weeks after cell transfer. Even after 40 weeks following cell transfer a highly significant degree of protection existed among mice challenged with ten LD_{50} doses of the organism. As expected, aged mice were particularly susceptible to the infection; that is, the 14-day mortality among 120- to 125-week-old mice was 68.5% when challenged with doses as low as 10% of the LD_{50} dose for young adult mice, and infection was 100% fatal for 135- to 140-week-old mice when challenged with doses as low as 5% of the LD_{50} dose for young adult mice. When programmed spleen cells from young adult mice were injected into 131- to 136-week-old mice and then challenged 2 days, 2 to 4 weeks, or 10 to 12 weeks later with a dose of organisms 20 times larger than that which gave 100% mortality for 135- to 140-week-old mice, mortality was 17, 50, and 75% respectively. Finally, our preliminary data on the effect of "cell banking" indicated that programmed and normal spleen cells can be stored at −196°C without loss of their protective capacity.

These preliminary findings suggest that immunological rejuvenation of aged mice is feasible and hopefully offers a rational approach for clinical investigators.

5.20 PATHOLOGY OF AGED $BC3F_1$ MICE

Fumitoshi Chino

Knowledge of the frequency of spontaneous diseases associated with natural death is obligatory for the evaluation of the experimental results involving aged animals. The current study in progress is concerned with spontaneous diseases observed in aged $BC3F_1$ mice, reared in conventional (clean) and substandard (dirty) farms. The median life-span of these mice is about 30 months of age in both farms. To date, complete necropsies and autopsies were performed on over 300 mice ranging in age from 21 to 43 months. About 80% of the death was associated with neoplasias and 20% with nonneoplastic diseases. Of the various neoplasias, reticulum cell sarcoma (RCS) was most predominant; that is, about 50% of those with neoplasias had RCS. Thus, death of aged mice can be categorized into three major diseases: (1) RCS (40%), (2) non-RCS neoplasias (40%), and (3) nonneoplastic diseases (20%). Among non-RCS neoplasias there were three major tumors: hepatoma, pulmonary tumor, and fibrosarcoma of subcutaneous tissue. The major nonneoplastic diseases were cardiac insufficiency, infectious disease, and renal disease. No significant difference was recognized between sexes. These preliminary data also indicated that the housing condition had little influence on the frequency of diseases associated with death. However, among mice with a shorter life-span (<30 months) death was associated with neoplasias more often among those reared in a dirty than a clean farm, and vice versa among those with the longer life-span.

5.21 AGE-RELATED CHANGES IN THE NUMBER OF HEMATOPOIETIC STEM CELLS IN BONE MARROW AND SPLEEN OF $BC3F_1$ MICE

M. G. Chen

Previous studies by this group showed that the decline in the potential for antibody production in aging mice is directly related to the decrease in numbers of competent progenitor cells of antibody-forming cells.[1] Currently, investigations have been made into changes in the numbers of more primitive progenitor cells, the pluripotential hematopoietic stem cells as measured by the spleen colony technique of McCulloch and Till;[2] these spleen colony-forming cells (CFU) are probable precursors of the progenitors of antibody-forming cells

and the progenitors for the other hematopoietic cell types.

Control young adult $BC3F_1$ mice were 3 months of age, while the aged $BC3F_1$ mice ranged from 33 to 38 months of age. Assay of the total number of CFU per femur in the old mice compared with the young mice revealed that the old mice had about 25% fewer CFU per femur; the concentration of CFU per 10^5 bone marrow cells in the aged mice was approximately 50% of that of the young mice. Qualitatively similar findings were obtained in studies of old compared with young spleen cells. The fact that stem cell compartment sizes in old and young mice were not too dissimilar, though total organ cellularity increased with age by a factor of 2, poses many questions as to the kinetics of hematopoietic cell proliferation in aged animals.

Preliminary comparative studies on the content of bone marrow (or spleen) cells which clone in vitro in soft agar, the so-called agar colony-forming cells (ACFU) first described by Pluznik and Sachs[3] and by Bradley and Metcalf,[4] again show equivalence of total stem cell compartment size in the old and young femur though this stem cell concentration is reduced by more than a factor of 2 in the aged mice. An obvious extension of this type of study would be measurements of other dependent stem cell populations.

Experiments are in progress on the reconstitution of lethally irradiated young adult mice with syngeneic bone marrow or spleen cells from aged donors. Preliminary results suggest that 38 days post transplantation, bone marrow CFU from the aged mice attain a compartment size and concentration in the young recipient equivalent to that of the old donor.

Other studies are testing the generality of these findings in mice of other strains with different mean life-spans. Studies are also being initiated into in vivo and in vitro interactions of stem cells from old or young animals and different environments.

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5.22 THE NATURE OF IMMUNOLOGICAL DEFICIENCY IN AGING

G. B. Price[1] Takashi Makinodan

An immunological deficiency which is expressed as both a delayed and suppressed level of antibody response to antigen has previously been detected in aged mice.[2] We are currently studying the nature of this deficiency by reconstitution of old immunocompetent cells with either bone-marrow-derived or thymus-derived cells from young and old donors. This system uses lethally irradiated thymectomized recipients as "culture test tubes" for these two cell types, which are the cells needed to generate an immune response. Preliminary work indicates that both cell types, thymus-derived and bone-marrow-derived, are deficient in the old animal. The nature of this deficiency – whether genetic or environmental – is now being sought through experiments dealing with the cellular kinetics of these cells from old and young adult mice transferred into both young and old "culture test tubes." A third possibility which involves recognition of the two principal interacting cells is also being tested.

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

[2] T. Makinodan and W. J. Peterson, *Proc. Natl. Acad. Sci.* **48**, 234–38 (1962).

5.23 IMMUNOLOGIC ACTIVITY OF AGED MICE REARED IN CLEAN AND DIRTY FARMS

Takashi Makinodan Bobbie S. Brewen
W. J. Peterson

Our earlier serologic studies[1] showed that the antibody-forming capacities of mice decreased to a level of about 10% of the young adults. These studies were extended over a period of about ten years, during which time dramatic improvements were made in the operation of animal care. Thus, by today's standard, a conventional mouse farm of a decade ago would probably be rated as a substandard farm. We do not know to what extent improvement in animal care may have influenced our findings.

Since studies on the effect of substandard environment on life-span and immunologic activity of mice have never been made, we decided to undertake this problem three years ago. "Specific-pathogen-free"

$BC3F_1$ mice, five to six weeks old, purchased from Cumberland View Farm, were reared in either a conventional (clean) or a substandard (dirty) farm. The diet was the same. Those in the clean farm received clean pans twice a week and chlorinated water (16 ppm) three times a week, and those in the dirty farm received clean pans only once a week and tap water twice a week. Viral, bacteriological, and parasitological tests at intervals showed that mice reared in the clean farm were positive for six of seven viruses tested (i.e., positive for Reovirus type 3, pneumonia virus, encephalomyelitis virus GDVII, polyoma virus, Sendai virus, and hepatitis virus, and negative for adenovirus), negative for external and internal parasites, and practically free of *Salmonella* and *Pseudomonas*. In contrast, those in the dirty farm were positive for external and internal parasites and for six of seven viruses tested. The water bottles in the dirty farm were purposely contaminated with viable *Salmonella* and *Pseudomonas* soon after we initiated this project.

Both primary and secondary antibody-forming capacities were assessed in terms of serum hemagglutinin and hemolysin titers and in terms of the maximum number of direct and indirect hemolytic antibody-forming cells in the spleen after an intraperitoneal injection of 10^9 sheep red blood cells (RBC). The secondary responders were immunized initially with 10^9 sheep RBC one month before the secondary immunization. The results revealed that no significant differences existed among the primary responders. However, among the secondary responders the females reared in the dirty farm were slightly superior to those reared in the clean farm. No striking difference in the life-span was noted; for example, the median life-span was comparable. These results indicate that "specific-pathogen-free" postweaning mice can adapt themselves to a substandard environment without any long-term effects on their immune competency and life-span. They further indicate that the infective bacteria may be acting as a weak adjuvant among the secondary responding females. To expand on these preliminary studies, immunologic and life-span indices are being assessed among young adult and aged mice bred in their respective farms.

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5.24 RELATIONSHIP BETWEEN IMMUNOLOGIC ACTIVITY AND PATHOLOGY OF AGED MICE

Fumitoshi Chino Takashi Makinodan

Previously we have shown that in aged mice the antibody-forming capacity, which reflects primarily the number of immunologic units, is reduced to levels as low as 10% of that of young adult mice. The current study is concerned with the relationship between immunologic activity of aged mice, general pathology, and pathology of the spleen. $BC3F_1$ mice of both sexes were reared either in a conventional (clean) farm or a substandard (dirty) farm. At 30 months of age, both primary and secondary immunologic activities were assessed in terms of the maximum number of direct and indirect plaque-forming cells in the spleen following an intraperitoneal injection of 10^9 sheep red blood cells. The immunologic activity was inversely related to the spleen size; with increasing spleen size, there was an increasing likelihood of reticulum cell sarcoma (RCS) invading the spleen follicles. We further noted that the immunologic activity was significantly lower among mice with atrophy of the spleen follicles and those whose spleen follicles were invaded with RCS. No significant difference in immunologic activity was observed between mice without tumors and mice with non-RCS tumors.

5.25 EFFECT OF THYMECTOMY ON THE LIFE-SPAN OF MICE

Takashi Makinodan W. J. Peterson
Fumitoshi Chino

Two years ago a series of studies was initiated on the effect of surgical, physical, and chemical insults on the immune system of mice, on their immune competence in later life, and their life-span. Such studies were made because, although it is known that the immune system of young mice exposed to most of these insults recovers rather rapidly, there is no information on the long-term effects of these insults. Of the various experiments initiated, sufficient data are available only on that involving thymectomy, and this is only partially complete.

Conventional mice were thymectomized when they were 2 to 26 months old. Their controls were sham operated. About 40 mice were involved in each group. Most of the surviving mice are now slightly over 30 months old. No attempt has been made in this initial

experiment to assess the immunologic activity of these mice. This will be done in our subsequent study. Necropsies have been performed on about 50 mice. The results indicate the median life-span in these thymectomized mice is not significantly different from that of the untreated controls. Furthermore, the frequency of death associated with infectious diseases appears to be higher than that of the untreated control; that is, 40 vs 20%. However, the sampling size is too small to generalize at this time the possible role of thymus in the defense against infections.

5.26 CATABOLISM OF SERUM PROTEINS IN AGED MICE

R. P. Quinn J. M. Ellis

Previous studies with senescing mice have shown that their antibody-forming capacity is significantly lower than that of young adult mice. These studies were based on peak serum titers and peak number of hemolytic plaque-forming cells in the spleen. Further, the preponderant antibody produced in aged mice is of the IgM class. In order to clarify age-associated changes in immunologic capacity it is necessary to establish the rates at which gamma globulins and other serum proteins are removed from the circulation. Therefore, mice ranging in age from 4 to 150 weeks were injected with purified serum proteins labeled with radioiodine (^{131}I), and the disappearance of the labeled proteins from intact animals was followed by whole-body counting. Among 150-week-old mice a high proportion displayed rates of disappearance of mouse gamma globulin about twice that found in mice of ages 4 and 21 weeks. In the aged mice there is a high incidence of pathologic destruction of lymphatic tissue (e.g., reticulum cell sarcoma, atrophy). The possible relationship between the rapid rate of disappearance of gamma globulins and the pathology of the aged animals is now being studied.

5.27 DNA FRAGMENTATION WITH AGING

G. B. Price[1]

DNA fragmentation has long been postulated as a cause of aging. However, no systematic studies based on the hypothesis exist. This laboratory has conducted studies to determine the state of DNA with respect to fragmentation in tissues of aged mice (30 to 35 months old) as compared with young adult mice (2 to 3 months old). Fixed tissues are used as a source of template DNA, for which calf thymus DNA polymerase and ^{3}H-labeled nucleotide precursors are supplied. (This portion of the work was performed at the laboratory of Dr. F. J. Bollum, University of Kentucky). Assuming that labeling intensity is directly related to the number of 3′-hydroxyl termini, we can obtain an index of DNA fragmentation. Significant fragmentation in aged mouse tissues as compared with "background" labeling of young adult mouse tissue has been detected. Specifically, heart, brain, and liver in aged mice contain cells in which there is considerable fragmentation of DNA.

Reference

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

5.28 LOW (MEDIUM) LEVEL EXPERIMENT

All Members of Pathology-Physiology Section

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. Another objective is to obtain correlative data associated with late somatic effects of radiation and aging. Such information is necessary if we are to estimate with confidence the effects of low-level irradiation in the dose range approaching present maximum permissible levels.

Previous progress reports have described the experiment in detail. To summarize: Each week groups taken from a total of 120 ten-week-old mice are exposed to a single whole-body dose of 10 to 300 rads of gamma radiation at a rate of 40 rads/min. They are then maintained under rigorously controlled environmental conditions – to protect the mice from disease and to reduce the influence of other variables since the difference in results for control and experimental mice is expected to be small. Most of the mice are allowed to live until death occurs naturally. Some of the mice are killed at weekly intervals from 12 to 120 weeks of age (serial sacrifice group). All mice that die or are killed are examined grossly and microscopically.

There were no further reductions in the size of the experiment this year, but reductions in staff have reduced the rate of examination of materials from the experiment.[1]

The last mouse in the second stage of the experiment, which was a preliminary test of the experimental protocol, died during the year. Processing of the data

from this group is now in the final stages. The last of the 15,000 mice required for the third stage of the experiment will be irradiated during spring 1970. About 7000 mice have now died, and the increase in mean cumulative mortality, reported earlier, is still seen at all doses including the smallest, 10 rads. Insufficient numbers have died to allow analysis of the influence of radiation on specific diseases, but there is an apparent relationship of mortality from thymic lymphoma to dose, including 10 rads.

Reference

[1] The original experiment was planned for 40,000 females and 40,000 males, with dose ranges down to 5 rads. Budget cuts plus cost-of-living increases forced the experiment to be cut to 15,000 females and no males, with the elimination of the 5-rad group.

5.29 PRELIMINARY RESULTS ON THE EFFECT OF DOSE LEVEL ON RBE OF FISSION NEUTRONS FOR MOUSE LENS OPACIFICATION

E. B. Darden, Jr. K. W. Christenberry
M. C. Jernigan

This work is part of a comprehensive study of factors which influence RBE in a highly radiosensitive system, the mouse lens. Young adult mice are given whole-body irradiation with single doses of fission spectrum neutrons in the Health Physics Research Reactor, or with comparable doses of 300-kev x rays. Slit lamp examinations are made at appropriate intervals after irradiation and the degree of opacification assayed using established scoring criteria. The data obtained to date suggest that the RBE decreases slowly or remains constant with increasing dose levels above about 100 rads. At lower doses RBE shows a more pronounced inverse response down to the smallest dose examined so far, 2 rads, with the likelihood that the RBE continues to increase down to very low doses. These observations when completed should permit a reasonably valid extrapolation of the dose response and corresponding RBE to the threshold or zero dose to be made in this particular tissue and should yield some insight on low-dose response in mammalian systems in general.

5.30 EFFECT OF FISSION NEUTRONS ON PRENATAL DEATHS IN MICE

E. B. Darden, Jr. W. Friedberg[1]
C. D. Hanneman[1] D. N. Faulkner[1]

Secondary neutrons produced by cosmic rays contribute an unknown but possibly substantial component of the total dose that might be received by passengers and crew in an exposed supersonic transport during a solar proton event. The human implications and the lack of pertinent information on the effects of prenatal exposure to high-LET radiation in the mammal have prompted an investigation to determine an RBE of fission neutrons for irradiation injury to the mouse zygote and early embryo. In the experiments to date, seven-week-old virgin mice are given overnight mating opportunity. Animals showing vaginal plugs are irradiated the following afternoon with a single dose of fission neutrons (dose rate of 2 rads/min) or are given sham irradiation in the Health Physics Research Reactor. Shortly before term the mice are killed and examined carefully for live and dead fetuses and resorption sites. The results have shown an increase in prenatal deaths with the radiation doses used, 2, 5, 10, and 20 rads. Deaths in the postimplantation period occurred at all doses, while at the highest dose there was evidence of death prior to implantation as well. The preliminary results suggest that relative survival is an exponential function of dose.

Reference

[1] Civil Aeromedical Institute, Federal Aviation Administration, Oklahoma City, Okla.

5.31 INFLUENCE OF PRENATAL IRRADIATION ON GROWTH, LIFE, AND AGE-RELATED DISEASES IN RF MICE

G. E. Cosgrove J. W. Conklin[1] A. C. Upton[1]

In a previous experiment RF mice in utero at periods of $9\frac{1}{2}$ to $19\frac{1}{2}$ days after conception were exposed to 50 to 400 r of whole-body x ray delivered to the gravid mother.[2] Group survivors of those treatments demonstrated some variations in mean survival time, aging disease incidence, and clinical appearance when compared with groups of mice postnatally irradiated at selected times to one year of age.[3] Expansion of a portion of the prenatal irradiation experiment seemed worth while.

In the present phase, fetal RF random-bred mice of both sexes were exposed to 300 r of whole-body x ray administered to the mother at days $14\frac{1}{2}$ or $18\frac{1}{2}$ after conception. Controls were born to sham-irradiated mothers. In irradiated groups perinatal mortality was excessive, especially in the $14\frac{1}{2}$-day group. Many of these deaths were associated with extensive cerebral

hemorrhage. Approximately ten mice of each experimental group were killed at 12 ± 1 month of age for measurements and histologic examination. The remainder of the mice in each group were allowed to die of natural causes and were then necropsied. Table 5.31.1 indicates the major findings in each group. All irradiated groups showed life shortening, most pronounced in the 14½-day group. Radiation-induced lymphomas and leukemias of thymic and myeloid types tended to be increased moderately in incidences in irradiated groups, but total lymphoma/leukemia incidence was not increased. Ovarian tumors were induced in irradiated females, but lung tumors were not increased in either sex. The incidence of renal glomerulosclerosis was high in all groups including controls. Irradiated mice sacrificed at one year of age had lower body, brain, and testis weight and partial testicular atrophy, especially in the 14½-day group, and the total length of intestine was also reduced.

Comparison with the earlier reported experiment showed similarities in life shortening except that the accelerated mortality of the original 14½-day male irradiated group was delayed (190- vs 305-day mean age at death). The stunting of irradiated mice was similar. Microcephaly was not noted this time although brain weights were reduced, probably in part related to lower body weights. Leukemia/lymphoma induction was more pronounced this time. In the original experiment, marked perinatal mortality with cerebral hemorrhage was not prominent.

The radiation exposure used (300 r), even though administered after the period of organogenesis, resulted in profound delayed effects in the survivors; yet in terms of life shortening and leukemia induction as we usually see them in irradiated young adult RF mice, the dose response was less except in the case of life shortening in the 14½-day male group. The reason for this sex difference was not discernible. The experiments have not been large enough to give definitive, repetitive results.

References

[1] Present address: Department of Pathology, State University of New York at Stony Brook, Stony Brook, N.Y.

[2] J. W. Conklin and R. A. Popp, *Biol. Div. Semiann. Progr. Rept. July 31, 1966,* ORNL-3999, pp. 114–15.

[3] A. C. Upton, J. W. Conklin, and R. A. Popp, pp. 337–44 in *Radiation and Aging,* ed. by P. J. Lindop and G. A. Sacher, 1966.

5.32 SEX CHROMATIN AND X-CHROMOSOME REPLICATION PATTERNS AND INCIDENCE OF SEX CHROMATIN

R. C. Brown[1] K. W. Cole

Reported studies of DNA replication patterns of sex chromatin and X chromosomes in partially or non-synchronized cell cultures, which suggest that all or part of the "late replicating" X chromosome of female mammalian cells is the "genetically inactive" sex chromatin of interphase cells, have lacked precise temporal staging of a particular cell, and have not given a definitive comparison of the replication patterns of sex chromatin and X chromosomes *throughout* DNA synthetic period since cells with clearly defined X chromosomes were not used. Since we had clearly shown the replication sequence of the very distinct X chromosomes of the dog throughout DNA synthetic period,[2] we chose dog cell cultures (primary diploid kidney cell cultures) in which to precisely determine the replication pattern of sex chromatin, using autoradiography and cinephotometric methods, and compared its replication pattern with that of both the "early replicating" and the "late replicating" X chromosomes. One pulse-labeled (10′; ^{3}H-thymidine) cinephotographed epithelial cell culture (363 cells) was allowed to grow so that the precise temporal relation of pulse to mitoses (pre and/or post) was known for each cell. Pulsed or daughter cells were examined for nuclear label and sex chromatin presence and label. A second cinephotographed culture (194 cells) was "pulse killed" and examined. Studies to verify that the sequence of replication of the X chromosomes of kidney cells was like that which we had previously determined in bone marrow were performed as previously described for bone marrow.

Replication patterns of the X chromosomes of the kidney cells in culture were like those previously described for bone marrow cells. The 66 labeled sex-chromatin-positive cells observed had time and intensity of sex chromatin replication precisely like that of the "late replicating" X chromosome throughout the DNA synthetic period. Sex chromatin prevalence increased with time following mitosis and was different in G1, S, and G2 periods (G1 < S < G2). Our studies suggest that sex chromatin, like the late-replicating X chromosome, begins "light" replication early in S period, increases in "intensity" of replication to maximum in mid S period and early part of late S period, and shows decreased intensity of replication near the end of late S period. This suggests that sex chromatin is

Table 5.31.1. Findings in Sham-Irradiated vs 300-r Intrauterine Exposure at $14\frac{1}{2}$ or $18\frac{1}{2}$ Days to RF Mice

Treatment Group	Sacrifice Group[a]				Survival Group									
	Weight			Length of Intestine (cm)	No. of Mice	Mean Age at Death (days)	Lymphoma Incidence (%)				Tumor, Lung (%)	Tumor, Ovary (%)	Mice with Neoplasm (%)[c]	Glomerulo-sclerosis (%)
	Body (g)	Brain (mg)	Testis (mg)				Thymic	Myeloid	Other[b]	Total				
Female control	36	467		51	94	637	2	2	50	54	21	6	70	67
Female 300 r at $14\frac{1}{2}$ days gestation	23	360		45	19	438	7	7	27	41	0	27	53	87
Female 300 r at $18\frac{1}{2}$ days gestation	28	408		47	41	484	10	2	38	50	7	22	73	70
Male control	34	453	95	54	97	681	2	2	49	53	21		69	66
Male 300 r at $14\frac{1}{2}$ days gestation	22	299	45	46	38	305	3	3	17	23	9		34	49
Male 300 r at $18\frac{1}{2}$ days gestation	27	402	58	49	49	453	7	7	34	48	23		61	66

[a]Killed at 12 ± 1 months of age, approximately ten mice per group.

[b]Principally reticulum cell sarcomas.

[c]Incidence of mice having one or more neoplasms at time of necropsy.

formed from the whole "late replicating" X chromosome rather than from only the late-replicating portions of the "late replicating" X chromosome. The observed incidence of sex chromatin in relation to the cell life cycle verifies some and refutes other reports of sex chromatin incidence in relation to the cell cycle. As have others, we observed replication of sex chromatin in condensed state; whether this reflects replication of noncondensed chromatin at the periphery of sex chromatin cannot be proven or disproven.

References

[1] Present address: Department of Pathology, University of North Carolina, Chapel Hill, N.C.

[2] *Cytogenetics* **5**, 206 (1966).

5.33 ASSAY OF MEGAKARYOCYTE PRODUCTION

T. T. Odell, Jr. C. W. Jackson

The objective of this investigation was to determine whether the uptake of tritiated thymidine (^{3}HTdr) by megakaryocytes in vivo could be used to assay the rate of megakaryocyte production. Rats were exchange-transfused with different volumes of platelet-poor blood until the peripheral platelet count was reduced to 8 to 66% of the initial platelet count. Twenty-four hours later ^{3}HTdr was injected intravenously, and one day after injection of the radioisotope the rats were killed and marrow smears were made. Autoradiograms were prepared with Kodak liquid emulsion, and the labeling index of megakaryocytes was determined on the Giemsa-stained autoradiograms. The labeling index of megakaryocytes was approximately proportional to the platelet count taken 1 hr after exchange transfusion. That of controls was about 38%, and that of rats whose platelet count was reduced to 8 to 10% of the initial count averaged 62%. Intermediate labeling index values were observed in rats whose platelet counts were between 10 and 100%. We conclude that this method is potentially useful for assessing rate of megakaryocytopoiesis.

5.34 SIZE CHANGES IN MEGAKARYOCYTES DURING MATURATION

T. T. Odell, Jr. C. W. Jackson T. J. Friday

In this study of megakaryocytopoiesis in rats, megakaryocyte size was related to ploidy and morphologic stage. Morphologic classification was made on Giemsa-stained squash preparations of tibial marrow. Thirty-five-millimeter pictures of the megakaryocytes in these preparations were enlarged on uniform-weight photographic paper, and the cells were cut out of the paper and weighed. The relative amounts of DNA in individual megakaryocytes were determined by the two-wavelength microspectrophotometric method after destaining the squash preparations and restaining them by the Feulgen reaction. Comparisons made with Student's t test showed that the average sizes of megakaryocytes differed between each pair of ploidy classes ($8N$, $16N$, $32N$) within a single maturation stage, or within a ploidy class between maturation stages (types I and II), with $p < 0.01$. There was thus a marked increase in size of megakaryocytes when moving either to a higher ploidy level within the type I (immature) compartment, or when moving at the same ploidy level from the type I to the type II compartment. Therefore, not only can megakaryocytes be separated into size classes that correspond to their morphologic stage of maturation, but also it is possible to distinguish size groups corresponding to ploidy classes within a maturation stage. The differences in size between the ploidy classes within the type I maturation stage are doubtless related in large part to differences in size of the nuclei of cells of different ploidy; in general, the type I megakaryocytes have a relatively small amount of cytoplasm. The marked differences in size of megakaryocytes having the same ploidy but differing in maturation stage (I and II) are, however, due to changes in the amount of cytoplasm. It is apparent, therefore, that megakaryocytes undergo a very active metabolic period between the time that DNA synthesis ceases and platelet formation takes place.

5.35 PLOIDY COMPOSITION OF MEGAKARYOCYTE STAGES

T. T. Odell, Jr. C. W. Jackson

Megakaryocytes in squash preparations of rat bone marrow stained with Giemsa were classified morphologically into three sequential temporal stages of maturation (types I, II, and III). The preparations were then restained with the Feulgen reaction, and determinations of relative amounts of nuclear DNA were made with the microspectrophotometric method. The results were used to determine the ploidy composition of megakaryocytes within the morphologic stages. In addition, tritiated thymidine was injected 30 min before the rats were killed to be able to distinguish in the Giemsa-stained autoradiograms which cells were in DNA

synthesis approximately at the time the marrow collections were made. The proportion of 8*N* megakaryocytes declined markedly in the successive maturation compartments, being 35% of type I cells, 15% of type II, and 7% of type III, while the relative proportion of 16*N* cells increased. The subgroup having the largest percentage of 8*N* cells (54%) was that comprising the labeled type I cells (in DNA synthesis). The percentage of megakaryocytes that were labeled was high in the low ploidy classes (73% of 4*N* cells) and declined with increase in ploidy level. These labeling results indicate that the recognized 4*N* megakaryocytes are turning over rapidly to supply cells to the succeeding compartments. In addition, many of the 8*N* cells are also replicating DNA to supply the 16*N* and, in turn, the 32*N* compartment, although some of them cease replicating DNA and differentiate into mature 8*N* megakaryocytes. In contrast, relatively few 16*N* cells and essentially no 32*N* cells replicate DNA, but rather undergo differentiation in the type II compartment. These findings clarify the movement of cells through the various ploidy levels and morphologic stages during the maturation of megakaryocytes.

5.36 CAUSE OF DEATH IN X-IRRADIATED GERMFREE RFM MICE

H. E. Walburg, Jr. G. E. Cosgrove

It has been repeatedly demonstrated that life-span is shortened by exposure to ionizing radiation in relation to the amount of radiation absorbed and that this action of radiation simulates an acceleration of the natural aging processes. It has been argued that a single whole-body dose of radiation should displace the Gompertz plot (age-specific death rate vs age) upward without changing its slope. Although some mortality data follow this prediction, some do not. The variable nature of these data may lie in differences in induction of specific diseases by radiation. To further define the role of specific diseases in radiation-induced life shortening and aging, germfree and conventional mice (both irradiated and unirradiated controls) were examined with respect to age and disease incidence at death.

Although it is difficult to establish the cause of death from histopathological diagnosis alone, it is obvious that certain diseases of the laboratory rodent reach an advanced state sufficient to disrupt one or more of the vital functions of the test animal. This is particularly true in the case of the inbred mouse strain RFM, where virtually all of the mice die from sequelae to one or more of five diseases. Furthermore, four of these five diseases are induced by a single whole-body exposure to x rays. An analysis of life-span in germfree and conventional mice reveals the following: (1) GF RFM mice live longer than their CONV counterparts, whether irradiated or not; (2) life-span-shortening effects of radiation are greater in female RFM mice than in males, whether GF or CONV.

An analysis of disease incidence at death reveals that radiation-induced leukemias (e.g., thymic lymphomas and myeloid leukemia) are not different in their combined incidence between GF and CONV mice, although the type of neoplasm expressed depends on the microbial environment in which the mice are reared. Removal from the sample of mice dying with these diseases reveals that very little life shortening is seen in the remaining sample of males, while considerable life shortening is seen in the remaining irradiated females. This sex difference can be explained either (1) by the fact that x radiation fails to increase the time of onset of severe nephrosclerosis in males while it does have such an effect in females or (2) by the rapid radiation induction of ovarian tumors in the female. In all cases, the life-shortening effects of radiation were quantitatively similar in GF and CONV mice, suggesting that although infections were common in the CONV mice, radiation damage to host defense systems plays little or no role in life-shortening effects of radiation in this strain.

To determine whether life-shortening effects of radiation can generally be ascribed to increased incidence of specific diseases, additional mouse strains with a wider variety of causes of death are presently being studied.

5.37 CAGE COVER EFFECT ON CAGE ENVIRONMENT

L. J. Serrano

Because of their success in reducing losses from diarrhea of infant mice in breeder colonies, protective covers for rodent cages hold the promise of a valuable defense against the transmission of disease in densely populated colonies of experimental animals. The cover is presumably a barrier to transmission of the causative agent; however, this has not been demonstrated. Further, there is a lack of information about the effect of these covers on the environment in the cage. This information is necessary if protective covers are to be used with experimental animals so that the user can balance the benefits against the risk to his experiment, can compare alternative methods for protecting his animals against transmission of disease, and, if he

chooses to use them, will know what additional variables need to be controlled.

To determine the effect of cage covers of the rod, wire-mesh, and fibrous-filter types on concentration of gases produced in the cage, we placed 4, 8, or 16 mice in cages of the shoe-box type and analyzed samples of air from each cage daily.

Protective covers, both filter and mesh types, had a major influence on the composition of air in the cage. They allowed the accumulation of CO_2 and NH_3 to levels much higher than in cages with open covers. When the mice were active or when the number of mice in the cage was doubled, the CO_2 level increased 50 to 100% above the minimal level determined by the protective cover. NH_3 was not detected until the third to sixth day, depending on the number of mice per cage, but by the seventh day reached noxious levels. Frequent changes of soiled bedding would eliminate the NH_3 hazard, and CO_2 levels could be reduced by limiting the number of mice in the cage.

5.38 EFFECT OF ANTIPLATELET SERUM ON RAT PANCREAS

W. D. Gude G. E. Cosgrove
C. W. Jackson T. T. Odell, Jr.

It was observed that rats injected with platelet-specific antiserum developed hemorrhagic lesions in the pancreas, whereas no lesions were observed in control animals similarly injected with normal rabbit serum. These findings raised the question whether this was a local effect of the antiserum on walls of pancreatic blood vessels or due to the general lowering of the platelet count in the circulation.

Hemorrhage was observed as early as 6 hr after a single injection of antiplatelet serum. The severity of the lesion had increased by four days. The lesion was characterized in its early stages by the presence of large numbers of red blood cells forming a ring around localized areas of acinar and islet cells; these rings were gradually replaced with large numbers of fibroblasts, many in mitosis. The long cytoplasmic processes of these cells revealed the presence of fibrin when special stains were applied. Subendothelial edema was also observed in some medium-sized arteries of the pancreas and spleen of experimental animals.

The occurrence and degree of severity of the lesions were correlated with numbers of circulating platelets. The stage of the lesions was related to the time after injection of antiplatelet serum. The most severe damage was observed in animals whose platelet counts were reduced to less than 1000 platelets/mm^3 for four days or more. (Platelet counts average about 1,000,000 per cubic millimeter of blood in control rats of the Sprague-Dawley strain.) Animals recovered from the pancreatic injury eight to ten days following a single injection of antiplatelet serum.

To determine whether the pancreatic lesion could be produced by means other than administration of antiplatelet serum, rats were given total-body radiation, which also lowers the platelet count. Identical lesions with hemorrhagic rings and fibrosis were produced in rats irradiated with 1200 r of x rays and killed eight to ten days later. Allowing four to five days for reduction of platelets, the lesion developed in the same time interval as was observed in rats receiving antiplatelet serum.

We conclude that a low platelet count in the peripheral blood is probably responsible for induction of these lesions in the pancreas after injection of platelet-specific antiserum rather than there being a direct action of antiserum on the pancreas, since similar lesions were observed after whole-body radiation, which also lowers the platelet count, but by a different mechanism. It is well known that platelets are necessary to maintenance of the integrity of blood vessel walls. To our knowledge, induction of such a lesion in the pancreas by a low circulating platelet count has not been reported previously.

5.39 EXPERIMENTAL THERAPY OF CANCER WITH RADIATION AND RADIOPROTECTIVE DRUGS

J. M. Yuhas J. B. Storer

In the treatment of cancer with ionizing radiation, normal as well as malignant tissues must be included in the radiation field, and the radiation dose which can be used is limited to that which can be safely tolerated by the normal tissues. The problem is further complicated with solid tumors, since their poor blood supply reduces their oxygen concentration and makes them more radiation resistant than the surrounding normal tissues.

Most efforts to resolve this difficulty have attempted to increase the radiosensitivity of the malignant tissues. An alternate approach has been attempted in our laboratory, namely, to increase the radiation resistance of the normal tissues. The method is based on the proposal that the poor blood supply of solid tumors should inhibit the rapid absorption of an injected radioprotective drug in much the same way that it causes reduction in oxygen tensions. Instead of allowing

the blood supply to pose a problem, this method uses it as an advantage.

Our initial studies[1] demonstrated that injection of 500 mg/kg of aminopropylaminoethylthiophosphate 15 min before exposure increased the radiation resistance of normal tissues (skin, bone marrow, and gastrointestinal epithelium) by 86 to 170%, but increased the resistance of a transplantable mammary tumor by only 15%. These data, in combination with pilot experiments in four spontaneous tumor systems, support the hypothesis that radioprotective drugs can provide an effective adjunct to radiation therapy.

We are currently developing a model system for studying the experimental radiotherapy of lung tumors. Lung tumors are induced in mice by injection of urethane. At appropriate times thereafter, when the lung tumors have arisen, the mice are treated with localized radiation, with and without the drug. If selective protection of normal tissues is obtained, as it was in the studies cited above, it should be possible to increase the effectiveness of radiation therapy of lung tumors. In addition, possible differences in the ability of normal and malignant tissues to convert aminopropylaminoethylthiophosphate to its protective free thiol form are being investigated.

Reference

[1] J. M. Yuhas and J. B. Storer, *J. Natl. Cancer Inst.* **42**, 331–35 (1969).

6. Carcinogenesis Program[a]

F. T. Kenney

Inhalation Carcinogenesis

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M. G. Hanna, Jr.

Immunology of Carcinogenesis

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Pathology and Virology

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Enzymology of Carcinogenesis

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Biochemistry of Carcinogenesis

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Biochemical Regulation

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Cytology and Genetics

E. H. Y. Chu

Mutagenicity of Carcinogens

F. J. de Serres
H. V. Malling

Somatic Cell Genetics

J. D. Regan

Repair Mechanisms

R. B. Setlow
J. S. Cook
J. D. Regan

X-Ray Crystallography

J. R. Einstein
C. H. Wei

[a]Research supported jointly by the National Cancer Institute, and the U.S. Atomic Energy Commission under contract with Union Carbide Corporation

[b]Postdoctoral investigator

[c]Leave of absence

6.1 EFFECT OF CHRONIC EXPOSURE TO ARTIFICIAL SMOG AND CHROMIUM OXIDE DUST ON THE INCIDENCE OF LUNG TUMORS IN MICE

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M. G. Hanna, Jr. R. F. Newell[1]
A. Hellman[1]

This inhalation study was initiated to determine the role of air pollutants on the induction of pulmonary tumors and to develop an experimental model in which the carcinogenic and cocarcinogenic activity (for the lung) of various agents can be tested. Chronic inhalation was chosen as the principal mode of exposure in order to induce pathophysiological processes similar to those that develop in the human respiratory system in response to inhalation of noxious and potentially carcinogenic chemicals. A major question underlying the experimental design was the following: Does an antecedent injury, such as influenza virus infection or x irradiation, modify the tissues of the respiratory tract and thereby increase their susceptibility to the tumor-inducing activity of inhaled, particulate, and gaseous chemicals?

Mice were exposed daily in inhalation chambers to either filtered air, chromium oxide dust, or ozonized gasoline; half of the animals in each chamber were exposed to 100 r x irradiation four weeks before the inhalation exposure was started. Another three inhalation chambers with the same agents were loaded with mice infected with PR8 influenza virus. In these three chambers also, half of the animals were x irradiated with 100 r whole-body irradiation. The main findings can be summarized as follows:

In C57BL/6 mice, males have a significantly higher incidence of lung tumors than females; irradiation raises the incidence of lung tumors in females but not in males; chronic exposure to artificial smog increases the lung tumor incidence in both sexes; infection with PR8 influenza virus appears to retard lung tumor growth (the differences found are statistically significant at the 5% level); exposure to Cr_2O_3 dust had no discernible effect on lung tumor incidence. No tumors other than adenomas and adenocarcinomas were found. Under the present experimental conditions combinations of treatments with the agents tested had no additive or potentiating effects. Comparison of our data with those reported earlier in the literature, in which similar experiments were performed, suggests strongly that infectious agents other than those tested might play an important role in the development of respiratory tumors. In the earlier studies in which a high spontaneous incidence of pneumonitis was observed, squamous cell carcinomas developed. In our own studies in which mice free of respiratory infections were used, only adenomas and adenocarcinomas were observed.

Reference

[1] Loanee; present address: National Cancer Institute, Bethesda, Md.

6.2 EFFECT OF ARTIFICIAL SMOG AND FERRIC OXIDE DUST INHALATION ON DEVELOPMENT OF DIETHYLNITROSAMINE-INDUCED LUNG TUMORS IN HAMSTERS

Paul Nettesheim M. G. Hanna, Jr.

Recent evidence suggests that the effect of compounds such as diethylnitrosamine (DEN) or benzo(*a*)-

Table 6.2.1. Hamsters. Chamber Operations Through December 31, 1969

Chamber	Date Started	Cotreatment	Chemical	Exposure (hr)	No. Animals	Total Dead
29[a]	3/26/69	None	None		135	6
30[a]	4/9/69	DEN[b]	None		135	7
31	5/7/69	None	Ozone, 1 ppm; gasoline, 90 ppm	961	135	10
32	5/21/69	DEN[b]	Ozone, 1 ppm; gasoline, 90 ppm	850	133	3
33	10/1/69	None	Ozone, 1 ppm; gasoline, 90 ppm; Fe_2O_3, 40 ppm	365	135	16
34	10/15/69	DEN[b]	Ozone, 1 ppm; gasoline, 90 ppm; Fe_2O_3, 40 ppm	236	135	27
35	6/11/69	None	Fe_2O_3, 40 ppm	835	135	3
36	7/2/69	DEN[b]	Fe_2O_3, 40 ppm	664	135	0
37	10/29/69	None	Filtered air	239	135	5
38	11/13/69	DEN[b]	Filtered air	140	135	7
Total all chambers					1348	84

[a] Nonchamber controls, kept in wire cages.
[b] 3 mg total, given in 12 weekly subcutaneous injections of 0.25 mg each starting at 6 weeks of age.

pyrene, which are carcinogenic for the respiratory tract, can be modified and enhanced by intratracheal instillation of inert "dust" or inhalation of SO_2 gas. These findings are of great importance because they suggest that noncarcinogenic air pollutants could play a major role in the development of lung tumors because of their potentiating effect. We selected hamsters to study the effects of chronic smog and ferric oxide dust inhalation on DEN-induced respiratory carcinogenesis, because hamsters develop respiratory tract tumors similar to those observed in man. Smog and ferric oxide are common air pollutants, and diethylnitrosamine is a potent respiratory carcinogen in the hamster.

The experimental design is summarized in Table 6.2.1. Groups of 135 male hamsters are injected weekly with 0.25 mg DEN subcutaneously (12 weekly injections, total of 3 mg DEN) and are exposed 6 hr/day, 5 days a week to an atmosphere containing either ozonized gasoline (artificial smog), Fe_2O_3 dust, or both of these agents given simultaneously. Control groups are either exposed to the same air contaminants without receiving DEN injections or receive DEN injections only. Macroscopic and microscopic investigations are performed on all moribund and dying animals. No respiratory tract tumors have been observed up to date, but are expected to develop within the next few months.

6.3 EFFECT OF PNEUMONITIS ON DISTRIBUTION AND RETENTION OF DUST IN THE LUNGS OF MICE

Paul Nettesheim R. F. Newell[1]

Many airborne carcinogens enter the respiratory tract as particulates or are carried on particulates. The factors controlling distribution and elimination of inhaled dust particles are therefore of great importance in studies concerned with the pathogenesis of lung cancer. Experiments in which specific-pathogen-free mice were chronically exposed to an "inert" dust showed that dust particles were very evenly distributed throughout the lung. However, when these animals were infected with a mouse-adapted influenza virus two weeks prior to dust exposure, heavy accumulations of particles in and around scars and in areas of interstitial fibrosis were observed. Chemical determination of chromium oxide content of infected and noninfected mice, one day to two weeks after termination of chromium oxide dust exposure, revealed that animals that had undergone influenza pneumonitis eliminated the chromium oxide more slowly than uninfected control mice. These experiments suggest that pulmonary infections might play an important role in the development of lung cancer, since particles which might carry carcinogens are more slowly eliminated from the lungs and since higher concentrations of carcinogens may be reached in injured lung tissue than in normal tissue.

Reference

[1] Loanee; present address: National Cancer Institute, Bethesda, Md.

6.4 DEVELOPMENT OF METAPLASTIC LESIONS IN THE LUNGS OF MICE AFTER TREATMENT WITH CARCINOGENIC AGENTS

Paul Nettesheim

A variety of mouse strains have been used as test animals in experiments concerned with respiratory carcinogenesis. The tumors observed in such studies are almost always pulmonary adenomas and adenocarcinomas. Only a few reports can be found in the literature describing the development of squamous metaplastic lesions and/or squamous cell tumors after administration of carcinogens. Recently the formation of a large number of squamous cell tumors in DBA/2 mice was reported after chronic administration of urethane.

We therefore decided to investigate whether the development of this type of lung tumor is dependent on the mouse strain, the type of carcinogen, or on the mode of administration of the chemical (i.e., long-term, as compared with short-term administration of carcinogen). In the first series of experiments, male and female DBA/2, BALB/c, and $BC3F_1$ mice were given ten weekly injections of urethane (20 mg per mouse per injection), or of 3-methylcholanthrene (at two dose levels: either 0.2 mg or 0.4 mg per mouse per injection). Serial sacrifices are performed beginning one week after the last carcinogen administration. The data obtained so far indicate that squamous metaplasias occur only after repeated urethane injections (not after 3-methylcholanthrene injections) and are seen only in DBA/2 mice. It appears that these lesions do not progress into tumors if the urethane administration is ceased after the ten injections; in fact, they seem to disappear with time. The only tumors observed so far are adenomas and adenocarcinomas. These preliminary data raise the possibility that the "squamous cell tumors" observed by other investigators after protracted urethane treatment may not represent true neoplasia.

6.5 APPEARANCE OF TRACHEOBRONCHIAL GLANDS IN AGING MICE

Paul Nettesheim D. H. Martin

In experimental studies of a variety of respiratory tract diseases, it has become of great concern to select a species similar to man in the occurrence and distribution of mucus-producing glands in the conducting airways. Rabbits and rodents such as mice and hamsters are generally considered to have no tracheobronchial glands.

In a study concerned with cocarcinogenic effects of chronic exposure to various environmental agents, we observed that aging mice ($^1/_5$ to 3 years old) had numerous glands or glandlike structures in their tracheobronchial tree. This prompted us to investigate the occurrence of such structures in various age groups (7 weeks to $2^1/_2$ years) of different strains of mice. No glands were found in numerous longitudinal and cross sections of trachea and bronchi of seven-week-old mice. In three- to four-month-old mice small single subepithelial glands were *occasionally* seen in the proximal half of the trachea. At seven months these were observed more frequently and sometimes appeared in small groups. At this time they were still extremely rare in the distal portion of the trachea and were never seen in the bronchi. In one- to two-year-old animals these glands were found regularly in great numbers in the proximal and distal trachea and in first- and second-order bronchi, and on occasion even in third-order bronchi. Many of them were cystic and distended with flattened epithelium and were filled with proteinaceous material, cell debris, and crystals. On PAS-staining, only a small number of cells contained PAS-positive droplets, but the material retained within those cystically dilated glands was very strongly PAS-positive.

The fact that these glands appeared in conventional, SPF, and germfree mice indicates that inflammatory processes are not a factor in their genesis.

6.6 CELL CHANGES IN RESPIRATORY EPITHELIUM DURING INFECTION: RELATIONSHIP TO LUNG CANCER

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

It has long been known that chronic respiratory infections in some animals result in significant cellular changes in the respiratory epithelium, specifically the bronchial epithelium. Of special interest in this regard are squamous metaplastic changes in bronchial epithelium of rodents suffering chronic respiratory infection(s). These changes have frequently led to misinterpretation of experimental results when the lesions were construed as neoplastic. Clinical experience usually reaffirms the infectious nature of the process; however, the histogenesis of the lesion has not been studied. More significantly, the etiology of the lesions has not been thoroughly studied, nor has the potential use of these findings in a controlled lung cancer experimental situation been reported. Studies currently in progress in mice aim at defining the sequence of morphologic cellular changes leading to the lesion of interest, and to gain a clearer understanding of etiologic agents and how they may interact to produce these changes. Possibly more than one agent is involved, but whether or not superimposition is necessary to produce the changes in the bronchial epithelium is not clear. Electron microscope studies have led to the discovery of a mycoplasma and a virus morphologically similar to pseudomyxo viruses. Furthermore, it is known that raw lung suspensions from infected mice will reproduce lesions characterized by metaplastic changes, but it is not clear that single infection with the above agents will. These studies are now in progress.

In addition to chronic changes in bronchial epithelium, chronic changes in alveolar epithelium are also seen. Cell kinetics involved in these changes and long-term sequelae are not well understood either. It seems quite probable that chronic inflammation may play a role in human lung tumorigenesis, but the area of abnormal lung physiology has not been fully studied.

6.7 TEST FOR MUTAGENICITY OF CHEMICAL CARCINOGENS IN MAMMALIAN CELL CULTURES

E. H. Y. Chu E. G. Bailiff

The purpose of this work is to test the somatic mutation theory of carcinogenesis by asking a simple question: whether certain chemical carcinogens or their metabolic products are mutagenic. We have tested, in the Chinese hamster cells, several carcinogenic and noncarcinogenic polycyclic hydrocarbons.[1] The possible mutagenic effect of these compounds was indicated by a forward mutational change from 8-azaguanine sensitivity to resistance.[2,3] Although with other groups of compounds there was a positive correlation between carcinogenicity and mutagenicity, we failed to show that such relationship exists for 1,2-benzpyrene (1,2-BP, noncarcinogenic) or 3,4-BP (carcinogenic). Neither compound was mutagenic. A recent study shows that 3-hydroxy-BP, a metabolic derivative of 3,4-BP, significantly increased the mutation frequency as compared with the control. In the

meantime, R. Kouri at the University of Tennessee has found that there is no benzpyrene hydroxylase in the Chinese hamster cells. These results clearly indicate that it is the metabolic derivative rather than the original compound per se that is effective.

A similar conclusion was reached when the mutagenicity test was made with an aromatic amine (2-acetylaminofluorene, AAF) and its metabolic derivatives. In the hamster cells, AAF was found not to be mutagenic. *N*-hydroxy-AAF (a proximal carcinogen) was slightly mutagenic, and *N*-acetoxy-AAF (the ultimate carcinogen) was highly mutagenic. Furthermore, we have evidence to show that the mutagenic action of *N*-acetoxy-AAF may be due to a base change from guanine:cytosine to adenine:thymine within the locus.

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6.8 IN VIVO INDUCTION OF CHROMOSOME ABERRATIONS WITH ALKYLATING AGENTS IN EHRLICH ASCITES TUMOR CELLS AND RAT BONE MARROW CELLS[1]

E. H. Y. Chu E. G. Bailiff

Tumor-bearing C3H mice, two to three months old, were treated intravenously with single sublethal doses of ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). Tumor cells, without prior colchicine treatment, were repeatedly sampled from the same animal and fixed at successive 12-hr periods following the chemical injection. In connection with a host-mediated study of mutations in *Neurospora*, cesarean-derived Charles River rats (Sprague-Dawley strain) were similarly treated with alkylating agents. After 1 hr exposure to colchicine, the rat bone marrow cells were removed for chromosome preparations.

There was an exceedingly low frequency of spontaneous chromosome aberrations in control animals treated with saline alone. In both systems, and at the maximum sublethal doses used, no chromosome aberration was induced with MNNG. On the other hand, both MMS and EMS effectively induced achromatic lesions (gaps), chromatid breaks, and exchanges in both types of cells. The frequency of aberrations increased with the amount of MMS or EMS administered and with time. The chromatid-type aberrations continued to appear in cell populations as late as 60 hr after treatment. No chromosome-type aberrations were found after MMS or EMS treatment.

The delayed and continued appearance of the chromatid-type aberrations in MMS- or EMS-treated cells and the absence of chromosome-type aberrations cannot be explained entirely by mitotic inhibition. It is possible that only subchromatid-type lesions were induced with these alkylating agents and that these lesions were not manifested at the chromosomal level until after DNA replication.

Reference

[1] Research supported jointly by the National Cancer Institute and the USAEC under contract with Union Carbide Corporation.

6.9 HOST-MEDIATED ASSAY SYSTEMS FOR MUTAGENIC ACTIVITY OF MUTAGENS AND CARCINOGENS

H. V. Malling

Many potent carcinogens do not react as such with the biological macromolecules in the cells but are metabolized into very reactive compounds (the ultimate carcinogens), some of which are mutagenic. The study of the internal level of mutagens is therefore extremely important for the understanding of carcinogenesis and mutagenesis in animals. The forward-mutation frequencies were measured in the *ad-3* region of *Neurospora crassa* by the direct method.[1] By using a forced heterokaryon between two biochemical marked stains, the diploid phase of higher organisms can be mimicked, and point mutations, as well as chromosome deletions, can be detected. Conidia from such a heterokaryon were injected into the peritoneal cavity of male mice (101 × C3H). Twelve hours later one of the following mutagens was injected into the tail vein: methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), and a monofunctional acridine mustard derivative (ICR-170). A significant increase in the mutation frequency was obtained after treatment with EMS and MMS under these conditions, but not after treatment with ICR-170 or MNNG. The mice therefore seemed able to detoxify MNNG and ICR-170 to a level at which mutations are not induced.

Reference

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6.10 ETHIONINE CARCINOGENESIS: THE INHIBITION OF RAT-LIVER METHYLASES BY *S*-ADENOSYLETHIONINE

Lawrence Rosen Shigemi I. Simms
G. David Novelli

S-Adenosylethionine (SAE), a stable liver metabolite of ethionine, inhibits the methylation of tRNA in vitro by normal rat-liver methylases.[1] Ethionine feeding also leads to increased tRNA methylase activity.[1-3] These findings suggest that either different methylases or more of the same methylases are synthesized by ethionine-fed rats in order to maintain tRNA methylation. The formation of new enzymes was suggested since methylases from ethionine-fed rats appear to be different than methylases from normal rats.[3] As a working hypothesis it was assumed that methylases of ethionine-fed rats are resistant to inhibition by SAE; this would help the animal to maintain the level of tRNA methylation during ethionine feeding. To test this hypothesis, we studied the in vitro effect of SAE on the methylation of *E. coli* tRNA by both preparations.

It was found that SAE inhibited the rate of overall methylation to the same extent with either preparation over a selected concentration range of SAE. A concentration of SAE was then chosen at which the overall rate was inhibited about 60%. Analyses of the methylated bases under these conditions were made to determine if the different methylases were inhibited to the same extent. It was found that both preparations inhibited the formation of 5-methylcytosine and of N^2-methyl- and N^2-dimethylguanine to the same extent, 50 to 60%, but that the formation of 1-methyladenine was inhibited more than 80%.

These results demonstrate that the methylases from normal and ethionine-fed rats do not differ with respect to SAE inhibition and that adenine-1-methylase is the one most affected by ethionine or its metabolite SAE. Its activity is the one most elevated due to ethionine feeding[3] and the one most inhibited by SAE. This suggests that ethionine feeding, leading to the formation of SAE, inhibits adenine-1-methylase; this causes more to be synthesized. This may also be true for other methylases, particularly cytosine-5-methylase.[3] It can be speculated that methylase synthesis is subject to feedback control; a possible regulator could be normal tRNA containing the particular methylated base. A first test of this will be to determine the endogenous level of methylated bases, particularly 1-methyladenine, in liver tRNA of normal and ethionine-fed rats and to relate this to methylase activity.

References

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6.11 ETHIONINE CARCINOGENESIS: ELEVATED RAT-LIVER METHYLASE ACTIVITY DUE TO ETHIONINE

Lawrence Rosen Shigemi I. Simms
G. David Novelli

The feeding of ethionine, a hepatocarcinogen, causes an elevation of rat-liver methylase activity. This has been demonstrated in vitro[1] and possibly in vivo.[2] Conflicting with the latter is the report that ethionine feeding leads to undermethylation of rat-liver tRNA.[3] The state of methylation of tRNA, whether increased or decreased, could have an effect on the biological activity of tRNA, and this might be related to the carcinogenicity of ethionine. For this reason, rat-liver methylase activity was compared in rats fed a normal diet (N methylases) and those fed ethionine (E methylases). The enzyme preparations were made before the ethionine-fed animals developed hepatic tumors. Assays were done in vitro with *E. coli* tRNA as substrate and methyl-labeled *S*-adenosylmethionine as the methylating agent. The methylase preparations were passed through G-75 Sephadex before use.

Preliminary results showed that the initial rate of methylation was two to three times greater with the E methylase preparation but that the extent of final methylation was greater with the N methylase preparation. Analysis of the methylated bases showed that about 90% of the total methylation could be accounted for by the following compounds: 5-methylcytosine, 1-methyladenine, N^2-methylguanine, and N^2-dimethylguanine. Small amounts of other bases could be detected. For this reason further experiments were done in which methylation was carried out for short or long times, and an analysis of the radioactive methylated bases in *E. coli* tRNA was made at each of these times. The short times, 10 and 30 min, were those in

which overall methylation was proceeding at a linear rate. Analysis at this time would be a measure of activity for the different methylases. The long time selected, 120 min, was chosen because the rates had leveled off.

Analyses at the short times showed that the increase in the activity of the E methylase preparation was mainly due to a 400% or greater increase in adenine-1-methylase activity and a 100% increase in cytosine-5-methylase activity. Increases in guanine methylase activity were slight, when observed.

The distribution of methylated bases at 2 hr showed that the amount of 1-methyladenine was about 100% elevated when the E methylase preparation had been used, but that the amounts of 5-methylcytidylic acid, N^2-methylguanine, and N^2-dimethylguanine were now higher when the N methylase preparation was used.

The differences between the two preparations for the rate and extent of methylation indicate that the enzymes are different although the same methylated bases are formed. These differences could be in their kinetic properties and/or site specificities; these would explain the results. Another important difference that must be considered is that there could be other endogenous compounds present which were not removed by G-75 Sephadex treatment and that these could affect the rate and/or extent of reaction. Further experiments involving mainly enzyme purification and determination of the position of the methylated bases in tRNA will be carried out to explain these differences and to determine their relevance to the carcinogenicity of ethionine.

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6.12 PRELIMINARY X-RAY DATA ON TWO CARCINOGENS, 5,6-DIHYDRODIBENZ(*a,h*)-ANTHRACENE AND 5,6-DIHYDROBENZ(*a,j*)-ANTHRACENE

C. H. Wei

The two (title) compounds differ markedly in their carcinogenic potency in rat skin painting, so that a comparison of their molecular structures should be of interest. Transparent needle-like crystals of both compounds, kindly made available by Dr. W. Lijinsky of the Eppley Institute for Research in Cancer, were recrystallized from *n*-hexane solutions. Crystals of 5,6-dibenz(*a,h*)anthracene (mol. wt, 280.37) are orthorhombic with cell parameters $a = 8.49$, $b = 11.65$, and $c = 15.14$ A. The volume of the unit cell is 1497 A^3. The observed density of 1.23 g/cm^3 (by the flotation method) is in good agreement with the calculated value of 1.24 g/cm^3 based on four formula units of $C_{22}H_{16}$ in a unit cell. Systematic absences of $0kl$ for l odd, $h0l$ for h odd, and $00l$ for l odd indicate a probable space group $Pca2_1$ (C_{2v}^5, No. 29). The second compound, 5,6-dihydrobenz(*a,j*)anthracene (mol. wt, 280.37), crystallizes with eight formula species in a monoclinic unit cell of dimensions $a = 12.16$ A, $b = 8.11$ A, $c = 30.71$ A, and $\beta = 100°50'$. The volume of the unit cell is 2975 A^3. The observed density of 1.22 g/cm^3 compares satisfactorily with the calculated value of 1.25 g/cm^3. Systematic absences of $h0l$ for l odd and $0k0$ for k odd are characteristic of the centrosymmetric space group $P2_1/c$ (C_{2h}^5, No. 14). Structural analyses of these closely related stereoisomers are under way.

6.13 THE EFFECT OF CUMULATIVE DOSE AND DOSE RATE ON DIMETHYLNITROSAMINE ONCOGENESIS IN RF MICE[1]

N. K. Clapp A. W. Craig[2] R. E. Toya, Sr.

Dimethylnitrosamine (DMN) is a potent carcinogen in a variety of species of animals and in the RF mouse induces lung adenomas and liver hemangiosarcomas in high incidences.[3] To determine the relationships between the total dose of DMN and induction of lung and liver tumors, 241 eight- to ten-week-old male RF mice were given DMN in the drinking water in daily doses of 0.4 (two groups), 0.9, and 1.8 mg per kilogram of body weight for treatment periods of varied length and with cumulative doses ranging from 87 to 243 mg/kg.

Incidences of lung adenomas in all treated groups were increased over controls by a factor of 2 and reached incidences of nearly 100%. While incidences of hepatocellular tumors were not affected, incidences of liver hemangiosarcomas were increased in higher dose groups and reached a maximum of 96%. The incidences of tumors varied with dose rate as well as total dose. In two groups receiving the same total dose (87 mg/kg), hemangiosarcomas appeared only in the group treated at the highest dose rate, suggesting that repair of damage by DMN may occur at the lower dose rate. Since lung tumor incidence was high in DMN-treated mice, metabolism of this chemical into a proximate carcinogen probably occurs in the lung parenchyma as

well as the liver. We found no shift in tumor site with variations in total dose as was reported in the rat.[4] The induction of tumors with two distinctly different cell types within the liver by DMN and DEN, hemangiosarcomas and hepatocellular tumors,[5] respectively, suggests that the cell type in which metabolism occurs may be specific within an organ and may be an important aspect of tumorigenesis by these chemicals.

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6.14 CONCERNING VIRUS INFORMATION

Stanfield Rogers

The possibility of adding synthetic sequences of nucleotides to virus RNA with enzymes, and having this RNA retain its infectivity and induce the synthesis of corresponding polypeptides in infected plants, has been demonstrated. The tobacco mosaic virus (TMV) system was used. More recently, it has been found that the new virus synthesized in plants infected with the modified RNA retains the modification and is replicated with the modified RNA that was added. When poly-A is added to the TMV RNA, polylysine can be isolated from plants after several sequential passages of the virus. It appears that we now have a cloned line of modified TMV which induces the synthesis of decalysine. Analyses are being carried out at Berkeley in the laboratory of Dr. C. A. Knight to find exactly how many adenylate residues are added to the end of this modified virus.

Having found that information can be added to the end of virus RNA and have its effect transmitted in multicellular organisms such as plants, efforts are being made to find whether more significant information than that directing the synthesis of polylysine can be attached and so transmitted. This information can be of two types: synthetic sequences or naturally occurring ones. Naturally occurring sequences seem easiest to obtain from viruses because of their limited information content. It has been estimated that the Shope papilloma DNA is only large enough to direct the synthesis of about six proteins. In conjunction with Dr. Stewart Riggsby of the University of Tennessee, enough Shope virus DNA was purified to attach to nitrocellulose and thereby hybridize specific virus-induced RNA extracted from the virus-induced papillomas. This RNA was then eluted and top-layered, using a Vinograd centerpiece, onto 1.70 density cesium chloride and centrifuged at 44,000 rpm. Using ultraviolet optics, four bands were found. In conjunction with Dr. Breillatt of the MAN Program and a much more sensitive monitoring system, four and perhaps five bands with a sedimentation velocity of around 8 were found. These bands were eliminated by mild alkaline hydrolysis prior to centrifugation, confirming that they were RNA. RNA extracted from normal skin did not hybridize in a detectable way with the Shope virus DNA. Efforts are under way to compare the RNA's that hybridize with the Shope DNA when derived from wild cottontail rabbit papillomas (which synthesize large amounts of complete virus), RNA's from domestic rabbit papillomas (which have large amounts of intact infective virus DNA but little or no complete virus as they do not make the protein coat), and RNA's extracted from the Vx-2 carcinoma originally derived from a Shope papilloma but after 40 transplant generations losing all traces of the virus. This should cast light on whether the cancer still contains some virus information.

We are in the process of using a larger-scale preparative system of isolating the individual bands. Efforts will be made to attach each of these to the tobacco mosaic RNA to find if synthesis of the virus protein coat subunits or the virus-specific arginase can be induced in the tobacco plant. Efforts will also be made using polio virus RNA. It is hoped that the attachment of these RNA molecules to one another can be accomplished through the use of a carbodiimide condensing reagent. This reagent was synthesized for us by Dr. Pal of the Nucleic Acid Chemistry Group.

In conjunction with Dr. Ray Tennant of the Pathology and Physiology Group, a simian adenovirus with what is reported to be an infective linear DNA is being obtained in enough quantity to do experiments with a DNA virus similar to those with the RNA described above. Dr. Hayes and associates at the Los Alamos Scientific Laboratories are in the process of synthesizing the DNA sequence for a small biologically active polypeptide, bradykinin. This sequence we hope to attach to the simian adenovirus DNA using the condensing reaction mentioned above or the enzymatic system of Goulian, Kornberg, and Sinsheimer.

The Shope papilloma virus is known to induce an arginase resulting in the systemic effect of a low blood

arginine in rabbits, mice, rats, and man. Two cases of argininemia have recently been reported ty Terheggen and associates of Cologne, Bern, and Antwerp. Virus has been purified in conjunction with the MAN program. The European group mentioned above plans to see if the genetic defect (presumably, a lack of arginase) can be corrected through infection of the patients with the Shope virus, which is known to be a harmless passenger in man. This laboratory is cooperating in running certain chemical and immunological tests as they become necessary.

6.15 HISTOPROLIFERATIVE EFFECT OF RAUSCHER LEUKEMIA VIRUS ON LYMPHATIC TISSUE: HISTOLOGIC AND ULTRASTRUCTURAL STUDIES OF LYMPHATIC GERMINAL CENTERS AND ITS RELATION TO LEUKEMOGENESIS

M. G. Hanna, Jr. A. K. Szakal R. L. Tyndall

Immunologic inadequacy is the primary condition associated with leukemogenesis. Impaired cellular and humoral immune capacity during virus-induced leukemogenesis in mice has been repeatedly demonstrated. This finding corresponds to histologic descriptions of major alterations in the stromal and parenchymal components of lymphatic tissue. It has been assumed that the leukemia-virus-mediated immune depression is a competition between the virus and the antigen for a common precursor cell. Thus, even though the role of the immune system in the development of the disease is uncertain, it is still important in the understanding of virus-mediated leukemogenesis. What is lacking in our understanding of this interaction is knowledge of the involuntary relationship between the immunologically competent lymphoid cell and the virus.

We have demonstrated that antigens concentrate and persist in lymphatic tissue germinal centers during an immune reaction. This localization of antigen occurs extracellularly in plasma membrane infoldings of unique dendritic reticular cells that form the stroma of the centers. It has also been demonstrated that these centers are the sites of proliferative expansion of immunologically competent cells. From studies in germfree mice a strong argument can be made for the causal relation between the antigen localization and the proliferation of parenchymal cells (immunoblasts) in the centers. Our data suggest that (1) antigen-retaining reticular cells provide an environment for the association of immunocompetent lymphoid cells with antigen and that (2) this is a provision for the proliferation and differentiation of parenchymal cells. Further, we have demonstrated large numbers of endogenous C-type particles localized in spleen lymphatic germinal centers. We felt that understanding the role of C-type particles in germinal centers is important in comprehending early virus-induced immune suppression in leukemic mice. Therefore, we have systematically examined on the histologic and ultrastructural level changes occurring in lymphatic tissue after Rauscher leukemic virus (RLV) injection. Special reference was given to germinal centers during the early phases of the leukemogenesis. We have recently completed these studies and summarize the results as follows:

Histologically, the singular distinct alteration in RLV-injected mice was the hyperplasia in germinal centers, injected 24 hr and continuing into what is described as "dissociative growth" of the center until it appears to occupy the entire lymphatic nodule. Between four and seven days after RLV injection, cells morphologically indistinguishable from those originally seen proliferating in the centers, and eventually in the entire nodule, were dispersed throughout the spleen red pulp. At this time these cells are closely associated with hematopoietic cells. This lymphocytic hyperplasia, characteristic of the normal immune reaction, also becomes a prominent feature in leukemogenesis in these mice. The main difference between the histologic aspects of lymphatic tissue changes in the Rauscher-virus-injected mice and most systematic immune responses is the persistent hyperplasia of the immunoblasts and their lack of differentiation into mature plasma cells. These findings are correlated histologically with antigen localization capacity of the stromal reticulum of germinal centers, as well as overall immune suppression.

On an ultrastructural level, C-type viruses were observed localized extracellularly in the plasma membrane infoldings of dendritic reticular cells, which constitute the stroma of germinal centers. The number of viruses in these centers increased markedly during the first ten days after injection. A most important aspect of this study was the identification in the spleen of immunoblasts participating in the replication of C-type particles. Budding of C-type particles from parenchymal immunoblasts of the germinal centers occurred as early as 24 hr after injection. By seven days the virus-replicating immunoblasts were detected in the spleen red pulp in close association with hematopoietic cells. It was suggested that the immunoblasts may, as a result of the sequential aspects of the events, provide a source of C-type virus to proliferating cells of the spleen red pulp, such as megakaryocytes and erythrocytic precursor cells. This suggestion is based on the concept of selective cell susceptibility to virus infection and

subsequent viral replication, based on proliferative activity of the cell and concentration of virus particles. Viropexis among these cells in the red pulp was also observed in later intervals of this study.

It is generally accepted that not all cells participating in replication of the oncogenic viruses have undergone or will necessarily undergo neoplastic transformation. One extremely important question is: Does the tumor cell emerge from the hyperplastic cell reaction that we have described? We know that this question may be difficult if not impossible to answer with the help of morphologic parameters alone. Nevertheless, for the following reasons we would like to suggest the immunoblast as a possible candidate for one of the neoplastically transformed cell types: (1) It has apparently undergone a maturation arrest with concomitant loss of normal cell function. (2) This cell type has undergone certain morphologic alterations previously attributed to leukemic cells; one such alteration is the occurrence of reticulin fibrils in the cytoplasm. (3) The cell participated in replication of C-type virus at all intervals studied. (4) The immunoblast-like cells have undergone certain antigenic changes by 20 days, resulting in their phagocytosis and degradation by the liver Kupffer's cells. It is acknowledged, however, that this last event could simply be a function of viral replication alone.

6.16 HISTOPROLIFERATIVE EFFECTS OF RAUSCHER LEUKEMIA VIRUS ON LYMPHATIC TISSUE: STUDIES IN NORMAL AND ANTIGEN-STIMULATED GERMFREE BALB/c MICE

M. G. Hanna, Jr. H. E. Walburg, Jr.
R. L. Tyndall

The depressed immune capacity associated with murine leukemia virus infection, especially the Friend and Rauscher type, is correlated with the selective ability of oncogenic viruses to replicate in immunologically competent progenitor cells.[1] Our studies have strongly suggested a selective replication of virus in rapidly proliferating cells of the non-thymic-dependent region of the lymphoid follicles which ultimately results in the lymphoblastosis characteristic of the Rauscher disease. The purpose of this present study was to test this observation by infecting virus into germfree mice which are devoid of germinal centers as a consequence of the low state of immunologic activity.

The germfree mice were a good test system since we have previously demonstrated, along with others, that these mice are not immunologically deficient, but merely antigenically unstressed.[2] Previous studies with germfree and conventional mice injected with ^{125}I-labeled human gamma globulin have demonstrated that antigen trapping and localization occur extracellularly in the plasma membrane infoldings of unique reticular cells in the non-thymic-dependent regions of follicles, and, subsequently, accumulation and proliferation of immunologically competent progenitor cells occur in this region, resulting in the development of recognizable germinal centers. This takes several days, however, in the germfree mice and corresponds to the growth phase of serum antibody response. Thus it was considered that splenomegaly of Rauscher disease would be augmented by the establishment of active germinal centers in the germfree BALB/c mice previously immunized with sterile sheep erythrocytes.

In both the germfree RLV-injected and the antigen-stimulated germfree RLV-injected mice, a two-phase growth of spleens occurred during the first 30 days after injection. No significant difference was detected in the first phase, which occurred to day 10, during which time the spleen weights were more than doubled in size in both experimental groups. A more rapid growth in weights occurred thereafter in both groups. However, this effect was much more pronounced in the SRBC-immunized mice. Between days 10 and 20 the spleens in this group had a doubling time of approximately four to five days. At 21 days after RLV injection, a 65% difference in spleen weight occurred between the SRBC-immunized and the normal germfree RLV-injected mice. Mice began to die from ruptured spleens in the former group at 22 days, while no deaths occurred in the normal germfree RLV-injected mice until after the 40-day time point was taken. A marked difference in spleen weight still occurred at 21 and 30 days. The splenomegaly study was confirmed by measurements of cell proliferation as assayed by the injection and measurement of ^{125}I-UDR, a thymidine analog which is incorporated specifically into newly synthesized DNA. The percentage of ^{125}I-UDR retained per spleen was markedly higher in the SRBC-stimulated germfree mice as compared with the normal germfree RLV-injected mice at 21, 28, and 30 days.

The results of these studies demonstrate the necessary if not essential role of antigen-retaining reticular cells and immunoblasts of germinal centers in the lymphoblastosis associated with the splenomegaly of Rauscher disease. In addition, morphologic results strongly suggest differential effects of the Rauscher preparation on the thymic- and non-thymic-dependent regions of the lymphatic tissue.

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6.17 HISTOPROLIFERATIVE EFFECT OF RAUSCHER LEUKEMIA VIRUS ON LYMPHATIC TISSUE: ALTERATIONS IN THE THYMIC-DEPENDENT AREAS INDUCED BY THE PASSENGER LACTIC DEHYDROGENASE VIRUS

M. J. Snodgrass M. G. Hanna, Jr.

Many parameters of the serologic effects of lactic dehydrogenase virus (LDV) in mice have been studied without successful histological correlation to a tissue lesion. A peak virus titer of 10^{9-11} ID_{50} units per milliliter of plasma occurs within 12 to 24 hr following intraperitoneal inoculation with LDV. A virus titer of 10^{4-6} ID_{50} is subsequently maintained throughout the life of the animals. Inoculation also results in concurrent tenfold increase in plasma lactate dehydrogenase, a substantial leukopenia (lymphocytopenia), which is inversely proportional to the virus titer, and a slight erythrocytopenia and depressed hematocrit. Recent evidence also indicates that infection with LDV causes significant delay in host rejection of allografts, a thymic-dependent or cellular immune reaction.

Lactic dehydrogenase virus is found as a contaminant of many murine virus and tumor preparations, especially those of the Rauscher and Friend type. It does not elicit an immune response but rather acts as an adjuvant, the mechanism of which is uncertain. Accordingly, the purpose of this study is to systematically evaluate, at both histologic and ultrastructural levels of resolution, the mouse splenic lymphoid nodules at early intervals following infection with Rauscher leukemia virus (RLV) which is contaminated with LDV. This was done in an effort to differentiate the biological functions of these two viruses and to define a tissue lesion noted in the thymic-dependent area of the lymphoid nodules.

Male BALB/c mice 11 to 14 weeks old were used. They were separated into four groups and injected intraperitoneally with RLV, prepared by animal passage, which also contained LDV as a contaminant; LDV followed by RLV; Rauscher leukemia virus prepared in tissue culture (ORTC), which does not contain an LDV contaminant; and LDV followed by ORTC. Tissues were removed at 6 hr and at one, two, and four days following injection and prepared according to conventional methods for histological and electron microscopic examination. Ultrathin sections were examined in a Hitachi HU11B electron microscope.

Lymphoid nodules of the mouse spleen appear as a homogeneous mass of lymphocytes and reticular cells of various types with a central artery and occasional peripherally located germinal centers. Thymectomy with x irradiation and special reticular stains have shown these nodules separable into a central zone populated with thymocytes and a cortical zone of lymphocytes of splenic germinal center origin. Following inoculation with RLV, LDV + RLV, and LDV + ORTC, a specific sequence of events was seen to occur in the central thymic-dependent area. Six hours following inoculation this zone appeared unaltered histologically, and only a very few LDV were seen ultrastructurally. These were located intracellularly in groups of two to four particles.

Twenty-four hours following inoculation the thymic-dependent area in experimental groups 2 and 4 appeared highly depleted of lymphocytes, and a few tingible body macrophages were present. LDV was seen intracellularly in larger numbers. Forty-eight hours after infection groups 1, 2, and 4 were all highly depleted of lymphocytes, and many more tingible body macrophages were present, indicating active phagocytosis of cellular debris. LDV was seen ultrastructurally to fill the intracellular space of adjacent reticular cells and their processes (Fig. 6.17.1). This interval represents a peak in the activity of macrophages and virus production; at four days the macrophages are gone, and very little virus can be found. During these same time intervals no changes occurred in the thymic-dependent area of tissues of group 3, those inoculated with ORTC. These results are consistent with the presence and absence of LDV. However, LDV was seen consistently in association with reticular cells, so it must be concluded that the cause of lymphocytic death is a humoral mechanism which is specific for thymocytes.

6.18 HOST IMMUNE MECHANISM INVOLVEMENT IN PLASMA CELL TUMORIGENESIS IN BALB/c MICE

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H. E. Walburg, Jr.

Plasma cell neoplasms of humans and mice have been used in the study of specific aspects of the immuno-

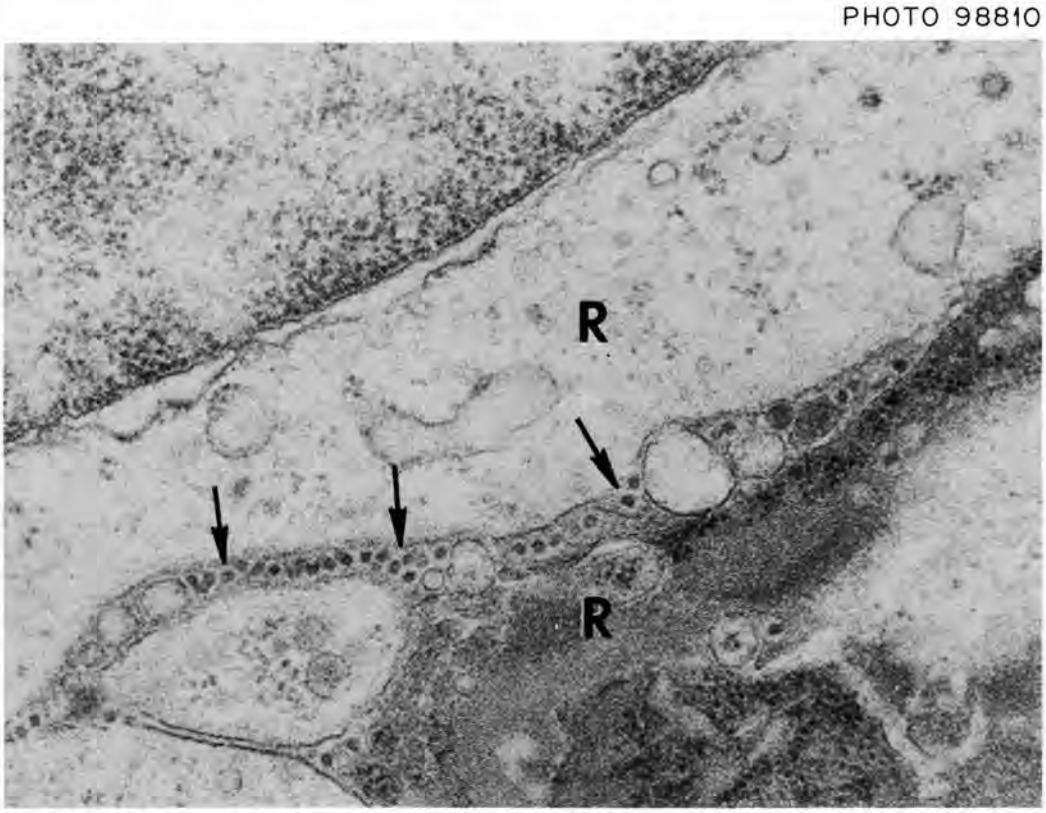

Fig. 6.17.1. Lactic Dehydrogenase Virus. Intercellular LDV (arrows) is seen localized between light and dark reticular cells (*R*) of the thymic-dependent area of a splenic lymphoid nodule in this electron micrograph two days following injection of LDV and Rauscher leukemia virus. 60,000X.

globulins produced by these malignancies and also for comparison of the tumor-secreted proteins with the products of the normal immune response. While the normal immune response, leading to the production of circulating antibodies, has been intensively studied, little attention has been paid to the pathogenesis of plasma cell tumors. In mice, plasma cell tumors are induced in the BALB/c strain by introduction of irritants such as plastics or mineral oil into the peritoneal cavity. The tumors appear months later, following the chronic interaction between host tissues and foreign material. BALB/c mice develop a high incidence (60 to 80%) of plasma cell tumor following the introduction of mineral oil in the peritoneal cavity. It has recently been shown that germfree mice, which have a less-developed lymphoreticular cell system, including a decreased number of plasma cells, than their conventional counterparts, when injected intraperitoneally with mineral oil fail to develop the expected incidence of plasma cell tumors. The incidence is less than 6%. This particular experiment suggests the importance of the microbial flora and/or the status of the immune system in the differentiation of plasma cells which respond to the carcinogenic stimulus in the genetically susceptible host.

The purpose of the present study was to stimulate germfree mice in such a manner as to induce germinal center activity in Peyer's patches of the intestine, and to follow this immune stimulation by mineral oil injection. Histologic and serologic results of alterations in the Peyer's patches as well as spleen and mesenteric lymph node in these animals will be compared with normal germfree animals stimulated with mineral oil alone. The present protocol allows for killing stimulated and normal mineral-oil-injected germfree mice at weekly intervals for the first three months. A second germfree isolator will be started so that animals can be held for long-term studies (eight to nine months) in an attempt to measure the development of plasma cell tumors. Further reports of these studies will be forthcoming in the next progress report.

6.19 THE EFFECT OF ANTIGEN COMPETITION ON BOTH THE PRIMARY AND SECONDARY IMMUNE RESPONSE IN MICE

M. G. Hanna, Jr. Leona C. Peters

The reduced immune response to an antigen as a result of a previous closely spaced reaction to a different antigen has been described in numerous studies. Recently this form of immune suppression (generally referred to as antigen competition) has been attributed to several mechanisms: a humoral factor generated during the immune response to the first antigen, this idea being compatible with the clonal selection theory of antibody formation; a competition by antigen for a limited number of immunocompetent precursor cells; and an effect by the initial immune stimulus, resulting in a deficiency of antigen-reactive cells available for processing the second antigen. Thus the mechanism in antigen competition is still quite equivocal.

Antigen competition studies have mainly been concerned with the primary immune response. Except for a few investigations, very few data have been compiled on the effect of antigen competition on priming. In a previous study we demonstrated that mice challenged with sheep erythrocytes three days after challenge with human gamma globulin developed a normal primary response but a depressed secondary capacity to the human gamma globulin. This suggests that a competition had occurred during priming mainly affecting the development of the sensitized cell compartment. We also suggested that the persisting levels of the two antigens, localizing in dendritic reticular cells of the spleen germinal centers, are possible factors in the competition for immune progenitor cells. The purpose of these studies was to obtain further information on the primary and secondary immune capacity in antigen competition.

Three antigens were used in these studies: sheep erythrocytes (SRBC), rat erythrocytes (RRBC), and human gamma globulin (HGG). The results of these studies demonstrated the following: (1) In both competition systems, SRBC prior to RRBC or HGG, a marked suppression of the secondary immune capacity corresponds to a suppressed primary agglutinin response to the second antigen. (2) The degree of suppression of the secondary agglutinin capacity can be reduced by increasing the dose of the second challenge, which suggests that in the secondary immune response there may be participation of both reacting sensitized and uncommitted immunocompetent cells. (3) The entire level of the immune suppression during competition is dependent upon the dose of the first antigen. Further, the experimental results do not support the possibility that the competition may be attributed to either an increased or decreased rate of antigen elimination. (4) Antigen competition cannot be attributed to a deficiency in the extracellular localization of antigen in the reticular cells of the splenic germinal centers. (5) The deficiency in a competition system is attributed to an impairment which ultimately affects the immunocompetent cell or unit. One reflection of this lesion on the antigen-sensitive unit is expressed in the competition between sheep erythrocytes and HGG. The detectable increase in the latent phase of the primary HGG agglutinin response occurs when HGG is injected at one or three days after SRBC. The recruitment of immunocompetent cells, and thus priming, is limited by the decreased level of preexisting tissue associated and/or free antigen. On the other hand, in a condition where there was limited immune progenitor cell recruitment to RRBC as a result of subsequent competition with SRBC, the result was antigen-driven depletion of the existing sensitized immune cell compartment. Relative to this finding, it was assumed that progenitor cell commitment to RRBC occurred normally for 48 hr. At this time the competition with a high concentration of SRBC successfully reduced progenitor cell recruitment to RRBC. With no difference in the amount of persisting RRBC antigen as a result of this competition, the sensitized cell compartment is depleted as a result of maturation to antibody-producing cells during the primary response. This depletion manifests itself as a reduced secondary immune capacity.

6.20 SPECIFIC INHIBITION OF IMMUNOCOMPETENCE

M. G. Hanna, Jr.

Studies with intact immunized animals have demonstrated that when the animals are rechallenged one to nine weeks after the initial immunization there is a period of depressed IgM antibody-forming capacity as measured by a direct hemolytic plaque-forming cell (DPFC) production which is below that of a normal primary response. This depressed DPFC, during a period when the total hemagglutinin levels are maximum, is difficult to interpret in the intact animal in view of current knowledge about antibody-mediated suppression.[1]

In response to sheep erythrocytes in mice, the hemagglutinin which persists during the primary response is primarily IgG, which has been demonstrated

to exert an inhibitory effect on IgM (DPFC) production. Our work, as well as the work of others, has suggested that this depression possibly represents an "exhaustion" of both the immune progenitor cell and the sensitized "memory" cell compartments and that IgM memory is short-lived.

Using the in vivo transfer method we have demonstrated a similar depression of the DPFC capacity at two and four weeks in primed mice. Because of the lack of interference from high levels of preexisting antibody in this transfer system, this depressed DPFC capacity at two weeks after priming can be considered to be a reflection not only of the lack of memory cells but also of the progenitor cell compartment. It is unclear, however, whether in this system we are dealing with the general depression of progenitor cells or a specific suppression for the priming antigen. The following experiments were designed to determine whether a normal primary level of DPFC's to a *different antigen* could be obtained during this period of reduced capacity. Male $BC3F_1$ mice were used in these studies. The assay system used was the hemolytic plaque-forming technique. Using the transfer system, donors were injected intraperitoneally with optimum dose of sheep erythrocyte antigen at 0 time. Irradiated recipients were then injected intravenously with 25×10^6 spleen cells from either primed or nonprimed donors, mixed with 25×10^7 sheep, rat, or horse erythrocytes. The numbers of DPFC's on days 4, 5, and 6 were assayed from spleens of the recipient mice. The DPFC measurements made at days 4, 5, and 6 after transfer show no real difference in DPFC's of either experimental group when compared with the controls. This conformity of response is measured in the presence of a weak cross-reactivity between two test antigens, rat and horse erythrocytes, as measured against sheep erythrocytes. The frequency of DPFC's to sheep erythrocytes increases with the primary response to rat and horse erythrocytes. This cross-reactivity cannot be detected against the less-antigenic rat erythrocytes when measured during a secondary response to sheep erythrocytes. Therefore it is reasonable to assume that this weak cross-reactivity between the erythrocyte antigens would not have interfered appreciably with these measurements.

The results of the present experiments were compatible with studies of heterologous erythrocyte antigen competition in intact animals.[2] These studies demonstrated that the period of depressed primary hemagglutinin or DPFC potential to a second erythrocyte antigen in mice primed with sheep erythrocytes is maximum between 2 and 4 days and reaches normal levels between 7 and 14 days. Our results, however, demonstrate further that there is a period of specifically suppressed DPFC potential to the priming antigen at a time when other immune capacities are normal.

These results suggest a depletion of the sensitized or memory cell compartment between one and two weeks after priming with sheep erythrocytes. This supports the idea that IgM memory is short-lived in this situation. As postulated in previous reports, the depletion of the sensitized cell compartment could be a result of antigen-driven maturation of the sensitized cells to functional antibody-producing cells, as postulated in the X-Y-Z immune cell maturation scheme. Further, during this interval, there is a lack of specific immunocompetent (DPFC) progenitor cells in the prime donor spleen cell pool. This depression of the primary DPFC potential two weeks after priming may be due to a reduction in the number of specific progenitor cells, or to a specific antibody-mediated suppression.

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6.21 RECOVERY OF ANTIBODY-FORMING CAPACITY IN GERMFREE MICE

Paul Nettesheim H. L. Walburg, Jr.
M. G. Hanna, Jr.

Previous studies in this laboratory demonstrated that germfree mice possess an antibody-forming capacity similar to that of their conventional counterparts.[1] In fact, it was observed that germfree animals consistently show higher serum antibody levels than conventional mice during the late phases of the response. We then proceeded to study the recovery rate of immunocompetence from an x-ray insult in germfree and conventional animals.

Germfree and conventional CF_1 mice were x irradiated with 400 r whole-body exposure (controls remained unirradiated). Groups of mice were injected with 10^8 sheep RBC at either one, two, four, or six weeks after x-ray exposure. At different intervals after antigenic stimulation, serum was collected for determination of anti-sheep RBC hemagglutinins, and the number of antibody-producing cells per spleen was determined with the direct and indirect hemolytic plaque assay. The results again showed that germfree

animals regularly maintain a higher serum antibody level during the late phase of antibody response. The rate of recovery of the early 19S antibody response was similar in germfree and conventional animals. On the other hand, the recovery rate of the 7S antibody response (mercaptoethanol-resistant antibody) was greater in germfree than in conventional animals. However, no consistent difference in the number of plaque-forming cells per spleen was detected between germfree and conventional animals, whether unirradiated or irradiated. Comparison of the results obtained with the serum antibody assay and the hemolytic plaque assay suggests the possibility that prolonged maintenance of high serum antibodies in germfree animals, particularly of the 7S type, may be due to either a slower catabolism of 7S immunoglobulins or increased production of high-avidity antibody in these animals.

Reference

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6.22 SUPPRESSION OF ANTIGEN LOCALIZATION AND RETENTION BY IMMUNOSUPPRESSIVE DRUGS

Paul Nettesheim Anna S. Hammons

We have previously shown that x irradiation not only reduces the pool of antibody-forming cell precursors but also interferes with the capacity of lymphatic tissue to trap and retain antigen, specifically in lymphatic tissue germinal centers. It was not known whether immunosuppressive agents other than x irradiation would have similar effects. We therefore tested the effect of actinomycin D, cyclophosphamide, and cortisone acetate at dose levels comparable in terms of immunosuppression with an x-ray exposure of 400 r on retention of ^{125}I-labeled human gamma globulin in various organs. The experiments revealed that all three chemicals markedly suppressed antigen localization and retention in spleen tissue and spleen lymphatic nodules in particular (as shown by autoradiography), particularly when the drugs were given prior to antigen administration. In contrast, antigen retention in the liver was not altered by these chemicals, indicating that the mechanism of antigen retention in the liver is different from that in the spleen. At the dose levels used, cortisone acetate was more potent than any of the other agents in interfering with antigen retention in lymphatic tissues, though it is known to be less effective in suppressing early 19S antibody production. Our findings support the idea that antigen retention in spleen lymphatic nodules is related to the late 7S antibody response.

6.23 A COMPARATIVE STUDY OF RETICULAR CELLS OF THE VARIOUS COMPARTMENTS OF MOUSE SPLENIC LYMPHOID NODULES

M. J. Snodgrass M. G. Hanna, Jr.

Lymphocyte populations of the central (thymic-dependent area) and cortical regions of splenic lymphoid nodules have been shown to differ in function, mobility, and sensitivity to various physical and biological stimuli. Similar zonal separation of lymphocytes is also seen in lymph nodes. More recently we have demonstrated differences in oncogenic and nononcogenic virus susceptibility of cells in these different regions. Experimental and histochemical studies indicate that there are also considerable zonal differences in the functions between reticular cells in the cortical and thymic-dependent areas.

$BC3F_1$ mice were used in this study. Their spleens were fixed in 10% neutral buffered formalin or glutaraldehyde at 2°C and prepared for histochemical and morphological study at both the light and electron microscopic levels of resolution. Snook's reticular stain indicates a particularly heavy concentration of fibers in the cortical zone and a distinct paucity in the thymic-dependent area (Fig. 6.23.1). Electron microscopic observations indicate that this also represents the distribution of the reticular cell population. Two distinct forms of reticular cells are seen. One has voluminous, electron-lucid cytoplasm and a large nucleus with peripherally disposed chromatin. The other has condensed dark cytoplasm with a few infoldings and disposed chromatin. Intermediate forms and interdigitations of cytoplasmic processes indicate that these represent different stages in the life cycle of these cells.

Reticular fibers in germinal centers, located in the cortical region, are also stained by Snook's method. Injection of antigen, sheep red blood cells, indicates that the more undifferentiated reticular cells form extensive plasma membrane infoldings and function in an antigen-trapping capacity for maintenance of the immune response.

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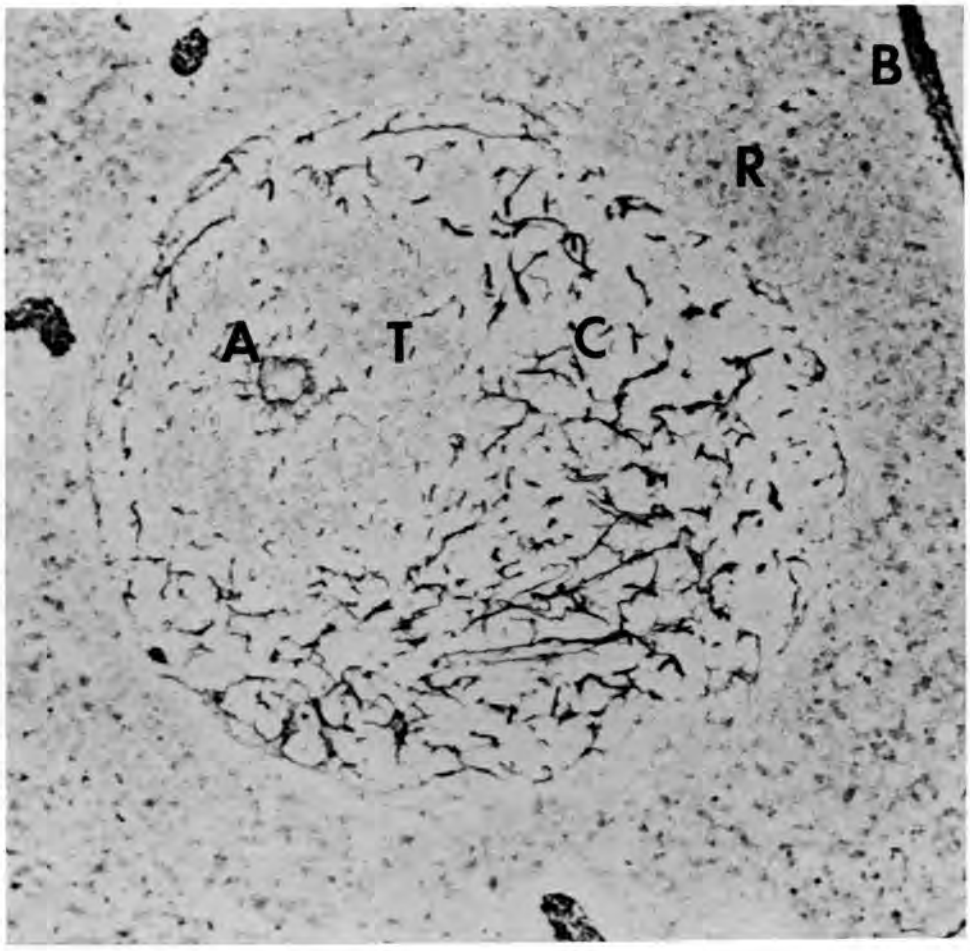

Fig. 6.23.1. Lymphoid Nodule. The relative distribution of reticular fibers in the cortical region (*C*) and thymic-dependent area (*T*) are seen with Snook's reticular stain. The central artery (*A*), red pulp (*R*), and several trabeculae (*B*) are also illustrated. 500X.

Preliminary studies of acid phosphatase and nonspecific esterase indicate that the reticular cells of the thymic-dependent area more closely approximate reticuloendothelial cells in terms of their function. This may be related to greater cellular mobility of this region. It was found also that lactic dehydrogenase virus replicates in these cells, but not in those of the cortical region or germinal center.

Based on morphological, histochemical, and functional studies of the reticular cells of the mouse splenic lymphoid nodule, there is a specific compartmentalization of the cortical and thymic-dependent areas. These studies also indicate functional differences between the reticular cells of these compartments. Those of the cortical region structurally support a less-mobile lymphocyte population and function in antigen retention during germinal center formation. There are fewer reticular cells in the thymic-dependent area, and they apparently have a more phagocytic function. Further results of this study, especially as the findings relate to such biologic aspects as virus cell susceptibility, spontaneous reticular cell sarcoma, and age-dependent changes in lymphatic tissue reticulum, are anticipated for future progress reports.

6.24 THE EFFECT OF AGE ON THE REGENERATIVE CAPACITY OF THE ANTIBODY-FORMING SYSTEM

Paul Nettesheim M. G. Hanna, Jr.

It is commonly thought that one major difference between the young and the aging organism lies in the

capacity to cope with and repair injuries. To test whether this notion applies to the antibody-forming system, ten-day-old, three-month-old, and two-year-old $BC3F_1$ mice were exposed to sublethal x irradiation, and the subsequent recovery of the serum antibody-forming potential to sheep red blood cell antigens was studied.

We found, as has been previously reported by other investigators, that the antibody-forming capacity of ten-day-old and two-year-old mice is below that of young adult mice, particularly with regard to the 7S antibody production. We also confirmed our previous findings that the 7S antibody response recovers more slowly from x-ray suppression than the early 19S response. Unexpected, however, was the finding that all three age groups revealed a comparable capacity to recover from 400 or 600 r whole-body x irradiation, regaining "normal" antibody-forming potential (i.e., relative to their unirradiated age controls) within five to six weeks after the x-ray insult. The present data do not permit a strict comparison of rates of recovery since they were obtained in the "intact" animal system. Experiments are being undertaken using the in vivo culture system of spleen cells to determine more precisely the rate of recovery of immunocompetence in the various age groups of mice.

6.25 ONCOGENIC EFFECTS ASSOCIATED WITH REPEATED INJECTION OF SHEEP ERYTHROCYTES IN BALB/c MICE

R. L. Tyndall J. A. Otten W. C. Kyker

Previous studies suggested that repeated injections of antigen might enhance expression of indigenous leukemia viruses. To test this possibility, thirty 12-week-old male BALB/c 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, during which time 11 of the mice died from causes unknown. One of five animals autopsied at cessation of treatment was diagnosed as leukemic on histological examination; the other four showed splenic hyperplasia. All remaining 14 mice subsequently died from leukemia 20 to 30 weeks after SRBC treatment. The leukemias were classified as reticulum cell sarcomas (RCS) and were transplantable.

In a second experiment, mice were similarly treated. None of these animals developed leukemia during 45 weeks of treatment. In a blind passage experiment, however, 1 of 20 animals inoculated at 13 days of age with spleen cells from an animal undergoing SRBC treatment developed an osteosarcoma two months after inoculation. A second animal, in the group of 20, developed leukemia five months after inoculation. No such malignancies occurred spontaneously in several hundred control animals in our facilities, nor in several thousand untreated BALB/c mice of comparable age in another laboratory (Dr. R. L. Peters, Microbiological Associates, personal communication).

The absence of leukemias in the second experiment indicates that the leukemogenic effects seen in the initial experiment are either not reproducible or are dependent on rather specific and as yet unknown conditions. The appearance of malignancies in 2 of 20 animals injected with spleen cells from an SRBC-treated animal in the second experiment, however, suggests that SRBC treatment may be eliciting an effect not necessarily expressed in the treated animals per se but unmasked by blind passage in young recipients. Even though results to date in this study are erratic, we feel that they are suggestive and important enough to warrant continued investigation.

6.26 STUDIES ON ACUTE LYMPHOCYTIC LEUKEMIA IN RATS

C. B. Richter

In contrast to the mouse, little or no evidence exists to prove the relationship of C-type virus to rat leukemias, yet rats are quite susceptible to leukemia viruses of other species. The leukemia under study here appeared spontaneously in three- to five-month-old Sprague-Dawley (SD) rats reared under closely controlled isolator conditions. This leukemia has since been carried serially by transplantation to preweaning SD rats. Studies to date using electron microscope observations suggest that if an associated C particle does exist, it is not ordinarily replicated by malignant cells. Furthermore, attempts to passage the tumor with entirely cell-free material have not been successful, indicating that either virus titers are very low or a virus is not involved. Alternatively, some host mechanism rejects virus infection or suppresses virus expression; however, this seems unlikely in view of the rat's susceptibility to other leukemia viruses. With viruses becoming more definitely associated with leukemias in other species, it seems unlikely that the rat system should be entirely independent and not have an associated virus. Studies now under way will determine if a viral relationship can

be established by systematic search for C particles, x-ray, or chemical induction of virus. Since relatively long-term solid tumor carriers can be established by subcutaneous transplantation, local and systemic immunological phenomena will be studied and related to other known leukemia viruses. It is hoped that if successful these efforts may help show the way to determine if viruses are related to human leukemias.

6.27 REQUIREMENT OF CELLULAR SYNTHESIS FOR RAT VIRUS REPLICATION

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

A study of the congenital and perinatal defects in rats or hamsters induced by rat virus (RV) led Margolis and Kilham to propose that the virus preferentially infected actively dividing cells. Subsequently we were able to demonstrate that in rat embryo cell cultures the efficiency of infection by RV was influenced by the level of cellular synthetic activity. Recently we have obtained more direct evidence of the actual dependence of rat virus replication on cellular synthesis. Since most chemical inhibitors of DNA synthesis involve complications of nonselectivity and reversibility, cells were irradiated prior to infection to specifically inhibit cell DNA synthesis. We found that x irradiation of rat embryo cells before infection impaired the ability of cells to synthesize the structural protein of RV, but did not impair their ability to synthesize pseudorabies virus. Synthesis of RV protein after graded doses of x ray, given before infection, yielded a curve which suggested that possibly two cellular functions are involved in rat virus synthesis. The first is a very radiosensitive function, and the second is relatively radioresistant. Ultraviolet irradiation of 10 ergs/mm^2 before infection impaired viral synthesis; above 200 ergs/mm^2 no viral protein synthesis was detected. Finally, treatment of cells with 5-fluorouracil prior to infection also inhibited synthesis of rat virus. Thus, the evidence which we have obtained shows that RV is dependent upon some cellular function(s) not required for the replication of other nuclear DNA viruses such as herpes viruses, adenovirus, polyoma, and SV40 viruses. Since RV contains single-stranded DNA with a molecular weight of about 2×10^6, the number of functions which the virus can specify is quite limited. The virus may, therefore, be dependent upon a cellular polymerase for synthesis of the complementary DNA strand for the replicative form of the viral nucleic acid.

6.28 ACTIVATION OF VIRUS SYNTHESIS IN NORMAL AND NEOPLASTIC TISSUE

R. W. Tennant R. E. Hand, Jr.
C. B. Richter Sally A. Thompson

It has been demonstrated that the induction of leukemia by x irradiation in C57BL and RFM mice etiologically involves a virus. The mechanism by which irradiation interacts with cells and the virus to produce the neoplasia is unknown. A crucial question is whether irradiation activates virus replication in cells which carry the viral genome in some repressed state, thereby permitting the virus to interact with target cells to initiate the neoplastic process. Our attempts to investigate this question involve the use of tissue cultures of embryonic cells derived from mice susceptible to radiation-induced leukemia. The experiments now in progress include treatment of the cultured cells with ionizing and nonionizing radiation and selected chemical carcinogens. The cells are examined for "activation" of virus synthesis by three methods: (1) the treated cells are examined for synthesis of viral protein using the Hartley-Rowe technique of fluorescent antibody assay; (2) the cells are examined for synthesis of progeny virions by electron microscopy; and (3) fluids from the treated cells are tested for leukemia induction in isogenic newborn mice. In addition, the sensitivity of these techniques is enhanced prior to assay by fusing the treated cells with sensitive indicator cells, using the virus-fusion technique of Harris and Watkins. If successful, these experimental approaches may be applicable to the search for viruses in human neoplasia.

6.29 PROTEIN AND ISOZYME CHANGES FOLLOWING INFECTION WITH THE RAUSCHER LEUKEMIA VIRUS

R. L. Tyndall R. C. Allen[1]
E. Friedman D. J. Moore[1]

An extensive effort has been made in the past two years to effectively separate isozymes of various normal mouse tissues and to compare the resultant patterns with those of the corresponding tissue from leukemic animals injected with the Rauscher leukemia virus (RLV).[2,3] Preliminary evidence in these studies indicated a similarity in profiles of fetal and leukemic spleen tissues, Efforts this past year strengthen this observation and suggest a hypothesis for the mode of action of the Rauscher virus.

Protein, lactic dehydrogenase (LDH), and esterase isoenzymes of leukemic tissues from mice infected with RLV were compared with the corresponding normal adult and fetal tissues. Similar comparisons were made between RLV-infected and noninfected JLS V6 and JLS V9 cell cultures. Cell-free extracts were separated electrophoretically in gradient acrylamide gel slabs using pulsed constant power. Microdensitometric analyses showed diminution of prealbumin and accentuation of postalbumin esterase activity in both fetal and leukemic thymus and spleen tissue. Leukemic and fetal thymus and spleen tissue also show increased activity in LDH isozyme V. Increased amounts of proteins with electrophoretic mobilities similar to serum albumin and transferrin were evident in extracts of fetal and leukemic thymus and spleen tissues. Isozyme patterns in the RLV-infected cell cultures showed alterations similar to that seen in the leukemic mouse tissue. These results indicate that infection with RLV results in the emergence of cells with LDH, esterase, and protein profiles similar to fetal thymus and spleen tissue. Whether by selection or direct conversion, the emergence of leukemic cells with fetal profiles following RLV infection suggests that the RLV infection results in a repression and activation of genetic information so as to give rise to cells of a fetal, dedifferentiated nature, that is, the leukemic cell. Studies are currently under way to determine the time course of these alterations following RLV infection and to characterize similarly the same parameters in cells infected with the Gross leukemia virus.

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6.30 ISOLATION OF PROTEIN COMPONENTS OF THE RAUSCHER LEUKEMIA VIRUS BY ACRYLAMIDE GEL ELECTROPHORESIS

R. L. Tyndall J. A. Otten Rother Johnson[1]

Protein profiles of leukemic tissue from mice infected with the Rauscher leukemia virus (RLV) indicated increased amounts of proteins electrophoretically similar to serum albumin and transferrin. In order to determine if such increase might be indicative of viral protein per se, protein from RLV was extracted and electrophoresed. Resultant protein bands were also tested for their immunogenic capacity for protecting mice against challenge with animal-passaged RLV.

Three hundred to four hundred milliliters of fluid from an established cell culture (JLS V5) infected with RLV was centrifuged at 1400 *g* to remove gross debris. The RLV in the clarified supernate was then banded isopycnically by centrifugation in a B-XIV rotor using a sucrose gradient. The banded, purified virus was pelleted at 50,000 *g* for 75 min. Viral protein was extracted from the resultant pellet with an ether–Tween 80 treatment and banded electrophoretically in acrylamide gel slabs. Staining for protein with Aniline Blue Black revealed the major protein component migrating similar to serum albumin with less prominent bands migrating as serum transferrin and haptoglobin. These results suggest that increases in albumin and transferrin-like protein in the extracts of leukemic tissue may be related to the presence of viral proteins. However, attempts to immunize mice with the different viral proteins against challenge with animal-passaged RLV were unsuccessful. Studies are currently under way to determine if the albumin-like protein in leukemic tissue extracts is the nonimmunogenic group specific antigen and also to determine what combination of purified viral or viral-induced antigens will successfully immunize against RLV.

Reference

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6.31 CHROMATOGRAPHIC ANALYSIS OF ISOACCEPTING tRNA'S IN RAT LIVERS AND HEPATOMA

Wen-Kuang Yang Kai-Lin Lee

Transfer RNA and aminoacyl-tRNA synthetases were prepared from the following materials: (1) adult rat liver, (2) mother and fetal rat livers, (3) two rat hepatoma cell lines, Reuber's H35 and HTC cells, and (4) regenerating rat livers after 70% hepatectomy. The tRNA was charged by the synthetases in vitro with either ^{3}H- or ^{14}C-labeled amino acid. Comparisons of isoaccepting tRNA profiles were performed by cochromatography in an RPC-2 column. The purpose of the study is to determine whether differences in isoaccepting tRNA's occur after hepatocarcinogenesis and, if so, what is the biological basis for the differences.

The results showed that the two hepatoma cell lines and the normal liver are different in their aspartyl- and tyrosyl-tRNA's. The rat liver contains two aspartyl-tRNA's, whereas both hepatoma cell lines contain four aspartyl-tRNA's, two identical to those found in the normal liver and two additional peaks. One or two tyrosyl-tRNA's, eluting relatively slower than other tyrosyl-tRNA's from the column, were found in the hepatoma but not in the normal rat liver. The characteristic aspartyl- and tyrosyl-tRNA's of the hepatoma were also found in the term fetal rat liver, although in much less quantity, but were not detected in the regenerating rat liver nor in the pregnant mother rat liver. This may indicate the appearance of fetal rat liver components in hepatoma tissues and that the altered tRNA patterns are not due to proliferating activity.

Since many tRNA's can be modified by the addition of an isopentenyl group to an adenosyl base and since hypothyroidism may deplete the formation of mevalonate and hence isopentenyl pyrophosphate in the liver, experiments were performed in which the effect of hypothyroidism on the tRNA profile was examined. Young male rats were thyroidectomized by surgical operation, followed by an injection of $Na^{131}I$ (0.5 mc per rat). Aminoacyl-tRNA synthetases and tRNA were prepared from livers of these rats one month later when signs of hypothyroidism became apparent. No alteration of chromatographic behavior, however, was observed with aspartyl-, tyrosyl-, phenylalanyl-, lysyl-, and seryl-tRNA's isolated from the liver of the hypothyroid rats.

6.32 ISOACCEPTING tRNA'S IN MAMMALIAN CANCER TISSUES

Wen-Kuang Yang

Our previous chromatographic studies with tRNA's from L-M cells and L-M tumors have demonstrated that in the L-M tumors aspartyl-tRNA's can be assorted into two duplex sets representing respectively the original L-M cell pattern and the "differentiated" cell pattern. Histochemical studies revealed remarkable functional features of connective tissue fibroblasts in the L-M tumors but not in the L-M cells. Furthermore, for a comparable transplantation, at least ten times more dispersed viable cells from the primary L-M tumors than L-M cells from original tissue culture were required. This indicates that a large portion of the cells in the L-M tumors might have lost their proliferating activity. All these findings, together with the reported information on the single cell transplantation with other tumor systems, suggest that cellular heterogeneity in the cancer tissue may in part be due to cell differentiation and that "stem cells" and "differentiated cells" in cancer tissue may contain different isoaccepting tRNA's.

A survey of RPC-2 chromatographic profile of aspartyl- and tyrosyl-tRNA's was carried out in various experimental tumors which included two different mouse plasma cell tumors, a fibrosarcoma and a lymphosarcoma from old BALB/c mice, mouse Ehrlich ascites tumor, and two rat hepatoma cell lines. All isoaccepting tRNA patterns obtained were consistent with the interpretation that the tumor consists of at least two cell kinds with different tRNA profiles, one similar to normal differentiated cells like liver and reticulocytes and the other having characteristics common to all of these tumors.

Preliminary results showed that the ascites form of MOPC 31C plasma cell tumor responded to chemotherapy of vinblastine sulfate or melphalan with a temporary regression of tumor size and a simultaneous decrease in the quantity of its "tumor type" isoaccepting tRNA's.

Reference

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6.33 CHROMATOGRAPHIC PROPERTIES OF tRNA ISOLATED FROM *E. COLI* UNDER CONDITIONS OF NUCLEIC ACID AND/OR PROTEIN SYNTHESIS INHIBITION

L. C. Waters

Quantitative and/or qualitative differences in isoaccepting tRNA's have been observed in the same organism under different physiological conditions. Whether these differences indicate a regulatory role for tRNA in cells is debatable. However, the central role of tRNA in protein synthesis and the multiplicity of isoaccepting tRNA's make it an attractive candidate as a regulator of cellular function at the translational level. One such observation of tRNA changes is that involving chloramphenicol (CAP) treatment of *E. coli*.[1]

The initial observations were: (1) tRNA synthesis continues after CAP treatment at a significant rate for up to 4 hr. (2) This tRNA accumulates and accepts leucine to the same extent as normal tRNA. (3) The chromatographic profile of leucyl-tRNA is drastically altered. We have pursued this investigation on the premise that this tRNA is in some way structurally

defective and as such might prove valuable in elucidating the functional significance of the base modifications known to be present in tRNA.

More recent data showed that CAP-tRNA accepts, in addition to leucine, phenylalanine and tyrosine to the same extent as normal tRNA. Also, these aminoacyl-tRNA's, as well as methionine and arginine, show altered chromatographic properties.

Studies with puromycin-treated relaxed and stringent strains of *E. coli* show that conditions which allow for continued RNA synthesis in the absence of protein synthesis are required for the appearance of the altered chromatographic properties. These data, the kinetics of appearance of the new phenylalanine tRNA peaks, and a combination experiment, in which rifampicin was used in conjunction with CAP, suggest that it is that tRNA synthesized after cessation of protein synthesis which chromatographs differently from normal. The fact that no new tRNA peaks appear when the stringent strain is deprived of the required amino acid or when rifampicin is used in combination with CAP argues that the new peaks are not modified forms of the tRNA present in the cells prior to treatment. Heating to 85°C did not eliminate the new peaks.

Efforts to determine the chemical difference between normal tRNA and CAP-tRNA, though not completed, have been negative. We have concentrated mainly on three of the modifications known to occur in tRNA at the polynucleotide level, that is, methylation, thiolation, and the addition of the isopentyl residue to an adenylic acid moiety.

In vivo methylation experiments with ^{14}C-methylmethionine and ^{3}H-uracil indicated that there were 8 to 25 times more methyl incorporated (relative to uracil) in CAP-tRNA than normal. However, experiments with ^{14}C-methionine and ^{32}P reduced this ratio to 1.5, and chromatographic analysis of the labeled nucleotides showed identical patterns for normal and CAP-tRNA. Therefore the methylation pattern of CAP-tRNA is not qualitatively different from normal and most probably does not differ quantitatively. CAP-tRNA does not accept methyl groups in vitro under conditions which methylate tRNA isolated from the methionine-starved relaxed mutant of *E. coli*. The phenylalanine tRNA profiles of CAP-tRNA and methyl-deficient tRNA are similar but not identical. Since the condition used to produce undermethylated tRNA is the same as that which can produce CAP-tRNA, with the added imposition of methyl deficiency, it is an interesting point as to whether it is the methyl deficiency or the CAP-related effect which is responsible for the altered chromatographic properties of undermethylated tRNA.

The possibility of the isopentyl modification being involved, though not examined directly, has been tentatively eliminated by two different kinds of data: (1) Permanganate oxidation, a process known to remove isopentyl residues, did not alter the chromatography of leucyl-tRNA. (2) Arginyl-tRNA, not believed to contain the isopentyl modification, shows a markedly altered chromatographic profile after CAP treatment.

Experiments to determine if thiolation is altered either quantitatively or qualitatively are under way. The basic idea behind this work is that, under conditions of protein synthesis inhibition, one or more of the modification processes become deficient and that the altered forms of tRNA which we observe are in some way structurally defective. As such, this tRNA might prove valuable in elucidating the pathway of tRNA modification and the biological function of such modifications.

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6.34 STUDIES ON THE PURIFICATION AND CHARACTERIZATION OF tRNA PYROPHOSPHORYLASE

Audrey N. Best G. David Novelli

We have extended our studies, described previously,[1] on the preparation of this enzyme. The enzyme preparation is free of tRNA nuclease, inorganic pyrophosphatase and ATPase activity at pH 9.5, alkaline and acid phosphatase activity with *p*-nitrophenyl phosphate as the substrate, several amino acid synthetases, and polynucleotide phosphorylase activity. The two detectable contaminants are a Co^{2+}- and Ca^{2+}-stimulated 5′-nucleotidase and a myokinase.

Studies on the substrate specificity of the tRNA pyrophosphorylase have revealed some unexpected properties of the enzyme. Two substrates of the enzyme, tRNApCpC and tRNApC, were prepared from mixed *E. coli* tRNA by periodate oxidation, amine cleavage, and alkaline phosphatase treatment. The normal reaction of the enzyme is to act as a cytidylyl- and adenylyltransferase and to produce the complete tRNA (tRNApCpCpA) from both tRNApC and tRNApCpC. We have found that the enzyme with CTP and either tRNApCpC or tRNApC will produce tRNApC$(pC)_n$pC, with greater internal CMP incorporation occurring in the case of the tRNApC substrate.

These abnormal products are not produced if ATP is included in the reaction mixture at five to ten times the concentration of CTP. Under optimal assay conditions, the K_m for ATP with tRNApCpC as substrate is 0.16 m*M*, while the K_m for CTP with tRNApC is about 0.04 to 0.05 m*M*. The abnormal product tRNApCpA is produced with tRNApC and ATP. We have some evidence that the presence of intact tRNApCpCpA will inhibit the formation of these abnormal products. Since there is so little known about the control mechanism of this enzyme, we believe that the ATP to CTP levels in the cell might be a controlling factor in the formation or maintenance of the normal tRNApCpCpA. These results have been verified using substrates from purified $tRNA^{Phe}$ and $tRNA^{Val}$. We have also found that UMP (from UTP) can be incorporated into tRNApC but probably not into tRNApCpC. This property has been reported for this enzyme from mammalian sources but not from bacterial sources.

We are completing this phase of the enzyme study (manuscript in preparation) and plan to explore various aspects of the cellular control mechanisms involved in the regulation of the activity of this enzyme.

Reference

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6.35 STRUCTURAL REQUIREMENTS OF STEROIDS TO INDUCE ENZYME SYNTHESIS

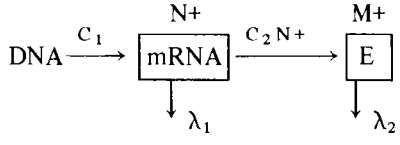
Kai-Lin Lee F. T. Kenney

A variety of steroids were tested for their capacity to induce tyrosine transaminase, or to compete with effective steroid inducers, in cell cultures of Reuber H35 hepatoma. Aside from compounds (including estradiol, estratriol, estrone, tetrahydrocortisone, and tetrahydrocorticosterone) which neither induce nor compete, the steroids tested fall into two groups. Steroids which do not induce but do compete with hydrocortisone include cortisone, prednisone, 17α-hydroxyprogesterone, 11α-hydroxyprogesterone, dihydroprogesterone, methyltestosterone, and testosterone. Steroids which do induce include corticosterone, aldosterone, 11β-hydroxyprogesterone, deoxycorticosterone, 20-hydroxyhydrocortisone, and progesterone. Relative inducing capacity (hydrocortisone = 100) was: corticosterone 100; aldosterone 90; 11β-hydroxyprogesterone, deoxycorticosterone, and 20-hydroxyhydrocortisone, 60; progesterone 7. The inducing steroids will also compete with hydrocortisone, and when added in excess will lower the hydrocortisone-induced enzyme level to that attained by the competitive steroid alone. We interpret these data as indicating a cellular receptor, to which steroids must bind in order to act. This binding requires the ketone group at position 3 and is facilitated by the double bound at position 4, and by the side chain at position 17, especially in the presence of the 20-ketone group. Binding is necessary but not sufficient for enzyme induction. To effect induction requires in addition the β-OH group at position 11; this can be replaced by H to yield a weak inducer. Addition of an OH group at position 21 will enhance the inducing ability. Studies are now in progress to identify the cellular receptor.

6.36 EVIDENCE FROM COMPUTER MODELING FOR TRANSCRIPTIONAL AND TRANSLATIONAL CONTROL IN HORMONAL REGULATION OF ENZYME SYNTHESIS

Kai-Lin Lee D. G. Hoel[1] F. T. Kenney

We have previously described[2] experimental evidence to support the conclusion that adrenal steroids induce enzyme synthesis by acting at the transcriptional level, while insulin acts on the translation step in the process of protein synthesis. Having obtained direct measurements[3] of the rates of degradation of our test enzyme, tyrosine transaminase, and of its mRNA, it was possible to construct a modification of the Jacob-Monod model as follows:



Knowing the absolute values for λ_1 and λ_2, values can be assigned to the other variables such that a steady-state level of M+ is maintained, comparable with the basal enzyme level observed in our cultures.

Given this situation it is now possible to ask what the kinetics of change in M+ will be when C_1, the rate of transcription, is increased by a factor of, for example, 10. The resulting computer-drawn plot almost exactly fits the observed kinetics of change in E after hydrocortisone treatment. In particular, the pronounced lag of about 1 hr is observed and is apparently due to the necessity of increasing the level of N+ (which is

undergoing turnover) before an effect on M+ is apparent. After this lag period M+ increases exponentially to approach a new steady state, as we observe after hydrocortisone treatment. If at this point the value of C_1 is reduced to its original value, the computer plot shows a period of 4 to 5 hr during which M+ declines slowly, reflecting the time required for N+ to be exhausted by turnover. After 5 hr M+ declines more rapidly at approximately the known rate of enzyme degradation (λ_2). These changes are again almost exactly those observed experimentally when hydrocortisone is removed from induced cultures.

When comparable manipulations are made with C_2, the rate of translation, the computer plot closely resembles our experimental results with insulin as inducer. In particular there is virtually no lag predicted (or observed), and M+ drops immediately at the rate λ_2 when C_2 is returned to its original value. These results show that there is no increase in N+, the mRNA component, when insulin is the inducer, while this component is increased after hydrocortisone treatment.

These analyses serve to confirm results gathered by other techniques from which we have drawn conclusions regarding the mechanisms of hormonal regulation in our test system. Perhaps more important, they also point the way toward determination of important parameters from kinetic analyses. For example, the lifetimes of mRNA's for other inducible enzymes (none of which are known) can be determined from the considerations given here.

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6.37 INTERACTION OF INSULIN AT THE CELL MEMBRANE AS THE INITIAL STEP IN ENZYME INDUCTION IN CULTURED HEPATOMA CELLS

Kai-Lin Lee

Since insulin can effectively induce tyrosine transaminase in cultured Reuber H35 hepatoma cells, we tested whether the initial phase of the induction may involve an interaction between the hormone and the cell membrane. The following evidence seems to support this concept: (1) Insulin covalently bound to a larger polymer, Sepharose (provided by Dr. D. Lockwood of Johns Hopkins University), will induce the transaminase, and the induction kinetics are similar to those with free insulin, and (2) addition of anti-insulin serum to the medium during insulin induction immediately stopped enzyme induction. These results suggest that there may be a specific intracellular effector, generated by a reaction of insulin at the cell membrane, which is responsible for enzyme induction. We are now in the process of testing for the existence of this intracellular effector.

6.38 REGULATION OF ORNITHINE DECARBOXYLASE

W. B. Panko F. T. Kenney

Ornithine decarboxylase is the first enzyme in the reaction sequence leading to the production of the polyamines spermidine and spermine, which are thought to play an important role in developing a cell's potential for growth. Preliminary work with this enzyme in two other laboratories indicated that ornithine decarboxylase (ODC) is located in the soluble fraction of rat liver. Partial hepatectomy initiates a very rapid and large-scale increase in the apparent level of the enzyme. A half-life of 10 to 12 min has been estimated for the protein, making it an ideal control point.

We have initiated a study of the enzymology of this enzyme in rat liver, preparatory to an analysis in depth of mechanisms regulating its level in the tissue and its role in stimulating growth. We have shown that ODC is a mitochondrial, rather than soluble, enzyme. New assay conditions have been developed, taking into account results of studies on parameters such as anion activation, buffer effects, and pH optimum. Our work indicates that previous workers were measuring less than 2% of the total ODC activity in the liver. We are now working out procedures to purify the enzyme and hope to develop immunochemical techniques to aid in analysis of its regulation.

6.39 STUDIES ON THE CONTROL OF PROTEIN TURNOVER IN RAT LIVER

R. W. Johnson F. T. Kenney

Individual proteins in rat liver possess remarkably heterogeneous turnover rates. Basically, four hypotheses have been advanced to explain the heterogeneity of turnover rates. The degradation rate is determined by:

1. the inherent stability of the protein,
2. ligand interactions, for example, coenzyme or substrate,

3. turnover of cellular organelle where enzyme is localized,
4. hormonal controls.

The first hypothesis predicts that a decrease in the stability of a protein molecule will be paralleled by an increase in the degradation rate, in vivo. This possibility will be tested by inducing tyrosine aminotransferase (TAT) in hepatoma cells in the presence of amino acid analogs. If TAT possessing the analog is less stable both in vitro, as measured by the rate of heat denaturation, and in vivo, as measured by its half-life, than "native TAT," this will provide support for the first hypothesis. Preliminary experiments indicate that an active enzyme is synthesized when *m*-fluorophenylalanine or 5-fluorotryptophan replaces the natural amino acids, phenylalanine and tryptophan.

Tyrosine aminotransferase requires the coenzyme pyridoxal phosphate. The holoenzyme is more stable than the apoenzyme in vitro. Attempts have been made to study the turnover rate of the apoenzyme and holoenzyme in hepatoma cells by growing cells on "low" or "high" pyridoxal medium respectively. This approach should provide information about the role of ligands in affecting protein turnover.

The time course of hydrocortisone induction of TAT in hypophysectomized rats and normal rats suggested that the turnover rate of TAT in hypophysectomized rats was slower. This suggestion was shown to be false by direct measurement of turnover rates in both types of rats using an isotopic-immunochemical method.

6.40 HYDROCORTISONE INDUCTION OF MULTIPLE FORMS OF TYROSINE AMINOTRANSFERASE FROM RAT LIVER

R. W. Johnson L. E. Roberson
F. T. Kenney

Three bands of rat liver tyrosine aminotransferase (TAT), following hydrocortisone administration, have been separated using CM-Sephadex chromatography at pH 6.0. A single chromatographic band is found with uninduced rats.

Multiple bands are never found with TAT isolated from Reuber H35 hepatoma. The hepatoma enzyme has been examined in uninduced cells, as well as in cells induced with insulin and hydrocortisone, alone or in combination. This rules out the possibility that multiple genes coding for discrete TAT molecules are responsive to the different hormones.

These multiple bands on CM-Sephadex have been found using liver 80,000 × *g* supernatants as well as with enzyme purified 40-fold. A single symmetrical peak is found if these bands are chromatographed on DEAE-cellulose, pH 7.6, DEAE-Sephadex, pH 7.9, or using gel filtration on Sepharose 6B, pH 7.0, or Sephadex G-100, pH 7.6. The latter observations indicate that aggregation or disaggregation phenomena are not responsible for the multiple forms observed only on cation exchangers and only on experimentation in vivo. The possibility that phosphorylation of the protein accounts for one or more of the bands was examined; no ^{32}P is incorporated into the enzyme. We will continue to investigate the significance of the multiple bands.

6.41 EVIDENCE THAT XERODERMA PIGMENTOSUM CELLS DO NOT PERFORM THE FIRST STEP IN THE REPAIR OF ULTRAVIOLET DAMAGE TO THEIR DNA

R. B. Setlow James German[1]
James D. Regan W. L. Carrier

Xeroderma pigmentosum (XP) is a recessively transmitted disorder of man that is characterized by increased sensitivity to ultraviolet light. It is inherited as an autosomal recessive, a mode of inheritance that suggests an enzymatic defect. Homozygous affected individuals, upon exposure to sunlight, sustain severe damage to the skin. This damage is characteristically followed by multiple basal and squamous cell carcinomas and not uncommonly by other malignant neoplasias. Regan *et al.*[2] showed that normal human cells could excise uv-induced pyrimidine dimers from their DNA, and Cleaver[3] showed that normal cells could do repair replication whereas XP cells could not. These results suggested that XP cells could not excise dimers and that the basic enzymatic defect is one of the steps in excision.

Our evidence indicates that the above suggestions are correct. A tissue culture cell line was derived from the skin of a man with XP. We labeled the DNA of normal and of XP fibroblasts with ^{3}H-thymidine, irradiated the cells with uv, and looked for the loss of dimers from DNA as the cells grew. Normal cells excise up to 70% of the dimers in 24 hr, but XP fibroblasts excise less than 20% in 48 hr. We also looked for the appearance of single-strand breaks in the DNA as measured by sedimentation in alkaline sucrose gradients. During the excision process in normal cells, single-strand breaks appear and then disappear as the gaps resulting from

excision are closed. Such changes are not observed in XP cells. XP cells apparently fail to carry out the excision process because they lack the required function of a uv-specific endonuclease, the nuclease that does the first step in repair. These findings indicate that unexcised pyrimidine dimers may be implicated in the oncogenicity of uv radiation.

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6.42 SELECTIVE UPTAKE OF GALLIUM-67 CITRATE IN LEUKEMIC MOUSE TISSUES

D. C. Swartzendruber[1] R. L. Hayes[1]
R. L. Tyndall

Gallium-67 citrate (^{67}Ga) has been shown to localize in many human tumors. The basic mechanism responsible for localization of this isotope in the malignant tissue is unknown. In order to study the factors involved in the uptake of ^{67}Ga by malignant tissues, appropriate animal models are needed. The degree of ^{67}Ga localization in spontaneous lymphomas in AKR and leukemic tissue in BALB/c mice infected with the Rauscher leukemia virus (RLV) was studied to determine if such diseases would serve as appropriate model systems.

Leukemic and normal adult AKR and BALB/c mice were injected intravenously with 2 μc of ^{67}Ga citrate. Twenty-four hours following isotope injection, the mice were sacrificed. The degree of isotope localization in whole blood, spleen, thymus, mesenteric lymph nodes, liver, kidney, lung, femur, and muscle tissues was determined by counting in a well scintillation counter. Results showed an eightfold increase in isotope uptake in spontaneous lymphoma tissue in the leukemic AKR mice compared with the corresponding normal tissue. Leukemic spleen tissue and blood from BALB/c mice infected with RLV showed two- and fourfold increases, respectively, in ^{67}Ga uptake as compared with the corresponding normal tissues. No significant differences were noted in ^{67}Ga uptake in the other tissues of normal and leukemic mice. These results indicate that these two types of murine leukemias may serve as useful models for investigating ^{67}Ga uptake in malignant tissues. Electron microscopic autoradiography studies are currently under way to determine the cell type(s) involved in the localization of gallium. It is hoped that these and additional studies will lead to a better understanding of the basis for ^{67}Ga uptake in human tumors and increase the diagnostic significance of ^{67}Ga localization in these malignancies.

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6.43 REGULATION OF THE SERINE REQUIREMENT OF HUMAN LYMPHOBLASTOID CELL LINES

Kathryn Lore[1] James D. Regan

We have previously shown that certain human lymphoblastoid cell lines have a requirement for a nonessential amino acid not ordinarily required for most human cell lines, namely, serine. Lymphoblastoid cells probably require serine because they derive from blood cells. In earlier work we showed that human leukemia cells and normal bone marrow cells in vivo have a serine requirement. Serine is required by these cells because their phosphorylated pathway of serine synthesis is repressed. The ability of single lymphoblastoid cells to form colonies in soft agar is greatly inhibited by the lack of serine. However, occasionally a few colonies appear. Several such clones have been isolated and have been maintained for protracted periods on serineless medium. The ability of these clones to synthesize serine from glucose and the activity of the enzymes of serine synthesis in these cells are under study. In any therapeutic approach utilizing nutritional deficiencies (e.g., asparaginase) in blood neoplasias, it is most important to determine how readily and by what mechanisms a nutritionally dependent leukemia cell can become independent of the nutrilite in question.

Reference

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6.44 NUTRITIONAL REQUIREMENTS OF BLOOD AND LUNG NEOPLASMS

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In our continuing studies of the nutritional requirements of human cancer cells we have extended our experiments to include two cases of lymphosarcoma

both of which displayed a serine requirement but no asparagine requirement. Phytohemagglutinin-stimulated normal peripheral leukocytes displayed this same requirement. Thus the reported sensitivity of peripheral leukocytes to asparaginase may be due to the glutaminase activity of this enzyme. Studies of glutamine requirements in the PHA-leukocyte system are in progress.

We have examined requirements for the in vitro nonessential amino acids in several specimens of human bronchiogenic carcinomas. No requirements for the seven nonessential amino acids were seen in these cells. In 48-hr experiments lung tumor cells exhibited essentially no DNA synthesis, while there was considerable RNA and protein synthesis. This is in contrast to our experience with leukemic cells in vitro, which always exhibited high rates of all three types of macromolecular synthesis. Apparently the lung tumor cells grow so slowly that essentially no DNA synthesis can be observed in a relatively short-term experiment. This result would suggest the mitotic poisons and DNA synthesis inhibitors would be relatively ineffective as therapeutic agents in lung cancer.

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