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HEMOLYSIN PRODUCTION IN IRRADIATED RABBITS

VIII. Colchicine Activity

W. H. Taliaferro and B. N. Jaroslow

Our studies to date[1,2] have demonstrated that the antibody-forming capacity of irradiated (400 r) rabbits can be completely or partially restored by injection of any one of several agents together with the sheep red cell antigen (1.6×10^9 RBC/kg). The data are summarized in Table 1. Restoration is probably related to the early radiosensitive period of the immune process, that is, the period which determines the amount of antibody formed and which eventually involves some aspect of the nucleic acid control of protein synthesis.

TABLE 1

The relative restorative activity of several agents on the hemolysin-forming capacity of irradiated rabbits.

	Complete	Partial	None
Tissue Preparations	Yeast extract HeLa cells	Rabbit spleen Mouse spleen	Rabbit kidney Rabbit muscle
Nucleic acid digests		DNA incorporated with DNase RNA incorporated with RNase RNase	Polymerized DNA Polymerized RNA DNase
Nucleic acid derivatives		Kinetin	Adenine Adenosine Adenylic acid Guanosine

The possibility of stimulation of cell division and of changes in cell populations is being studied with particular interest in the radiation-induced lengthening of the induction period. We tested colchicine (1.8 mg/kg), a mitotic inhibitor, in both irradiated and nonirradiated rabbits. The results are shown in Table 2. The restorative activity of colchicine in irradiated rabbits is exactly the same as that of yeast autolysate. Antibody titers are normal, but the induction period is not shortened as compared to that of the irradiated controls.

In nonirradiated rabbits, colchicine acts as an adjuvant if injected from 1 day before to 2 days after the antigen. Although yeast also has adjuvant activity, it is not nearly as marked as that of colchicine. This is graphically shown in Figure 1. Croton oil, injected subcutaneously as was

TABLE 2

Hemolysin response in rabbits immunized with a standardized suspension of sheep red blood cells.

Conditions ‡	Mean peak titer			Rate of accumulation (k_1)		Length in days of:		
	Log*	Geometric	Arithmetic	First phase*	Derived when >one phase*	Induction period*	Antibody rise to peak*	Number of rabbits †
1) Control: no X-ray	3.45 ± 0.11a	2,800	4,085 ± 865	0.97 ± 0.11a	0.67 ± 0.08a	3 0 ± 0.1a	7.4 ± 0.8a	14(2)
2) Control: X-ray only	2.18 ± 0.14A	150	209 ± 49	0.33 ± 0.11A	0.17 ± 0.14A	11 3 ± 1 2A	15.9 ± 1 9A	7(2)
3) Colchicine with antigen 1 day after X-ray	3.14 ± 0.13Da	1,400	2,400 ± 440	0.82 ± 0.10Db	0.30 ± 0.10Bd	10.1 ± 1.3Ad	11.3 ± 1.3Cc	18
4) Croton oil with antigen 1 day after X-ray	1.95 ± 0.15Ad	115	115 ± 28	0.49 ± 0.14Ad	0.30 ± 0.06Ad	11.8 ± 1.7Ad	10 ± 1.7Ad	4(3)
5) Croton oil with antigen	3.85 ± 0.20D	7,100	12,508 ± 5,800	1.31 ± 0.15D	0.78 ± 0.10D	3.2 ± 0.1D	8.6 ± 0.7D	8
6) Colchicine 1 day before antigen	3.85 ± 0.14C	7,100	10,030 ± 2,460	1.85 ± 0.23A	0.96 ± 0.07C	3.9 ± 0.2A	6.8 ± 0.6D	9
7) Colchicine with antigen	4.47 ± 0.07A	29,500	33,900 ± 5,700	2.86 ± 0.47A	1.16 ± 0.11A	3.2 ± 0.1D	6.7 ± 0.4D	11
8) Colchicine 2 days after antigen	4.33 ± 0.09A	21,400	25,400 ± 4,800	1.61 ± 0.12B	0.85 ± 0.07D	4.6 ± 0.1A	6.4 ± 1.1D	8(2)

‡ Colchicine and croton oil were injected subcutaneously, antigen was injected intravenously, and irradiated rabbits received 400 r whole-body irradiation.

*Data were compared to unirradiated controls when capital letters are used and to irradiated controls when lower case letters are used. A or a, $P \leq 0.001$. B or b, $0.001 > P \leq 0.01$. C or c, $0.01 > P \leq 0.05$. D or d, $P > 0.05$. Probabilities were not calculated for arithmetic mean peak titers and for those data not followed by an appropriate letter.

† Numbers in parentheses refer to additional animals that gave no antibody response or, as in groups 1 and 8, in which pre-peak titer serums were not available. Mean peak titers were calculated from entire groups, whereas the other measurements show the mean ± the standard errors for the antibody producers only.

colchicine, was used as a control to elicit an inflammatory reaction comparable to that of colchicine. It did not induce a significantly higher response in either of the antigen controls. Three possible colchicine mechanisms, alone or in some combination, are postulated: 1) Colchicine may act to synchronize the cell population so that there is a large population of potential antibody-producing cells in a state susceptible to antigenic stimulation. 2) it may stimulate the mitotic activity of antibody-forming cells at the end of X-ray-induced lengthening of the induction period. 3) It may act as a cytotoxic agent inducing karyorrhexis succeeded by liberation of nucleic acid degradiation products which interact with antigen as one of the early stages of antibody synthesis.

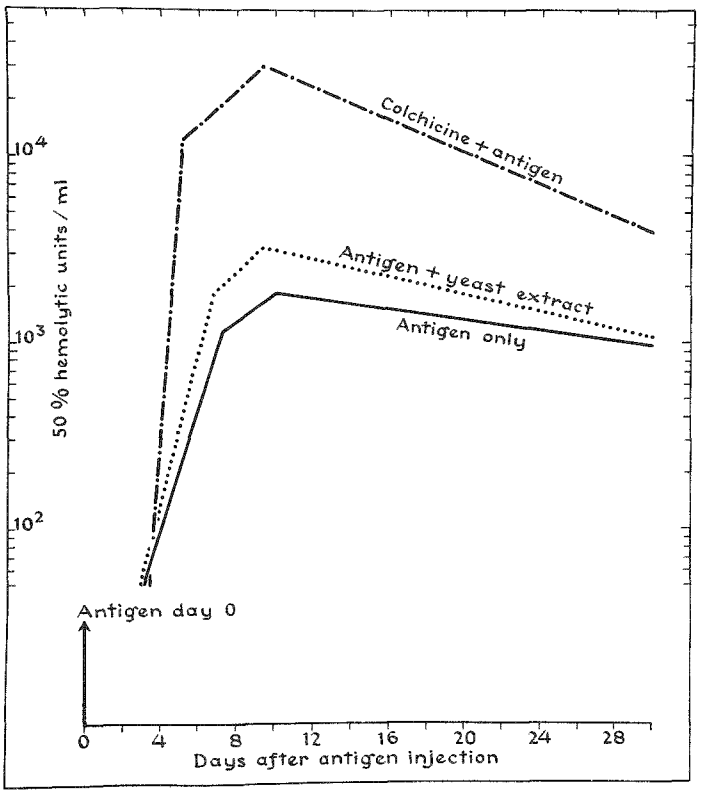

Figure 1

Typical hemolysin values of rabbits treated with yeast extract or colchicine.

References

1. Jaroslow, B. N., and W. H. Taliaferro. The restoration of hemolysin-forming capacity in X-irradiated rabbits by tissue and yeast preparations. J. Infectious Diseases 98 75-81 (1956).

2. Taliaferro, W. H., and Jaroslow, B. N. Restoration of antibody-forming capacity in X-irradiated rabbits. Proc. Second United Nations Conference on the Peaceful Uses of Atomic Energy. Geneva: United Nations, 1958. Vol. 23, pp. 79-83.

PROGRESS REPORT: PATHOGEN-FREE LABORATORY ANIMALS

Robert J. Flynn, Louise S Lombard, and Leroy O. Bibbs

Production Colony

The immediate objective of this project is to develop breeding colonies to provide an assured, disease-free supply of those strains of rats and mice commonly used by the Division of Biological and Medical Research. These rats and mice are to be free of those commonly occurring agents usually considered to be pathogens (Salmonella,pleuropneumonia-like organisms, Nelson's "virus," ectoparasites, and endoparasites); and they are also to be free of agents pathogenic under radiation stress (i.e. Pseudomonas aeruginosa). The ultimate objective is to develop breeding colonies to supply disease-free animals of all species and strains used by the Division.

All of the above-listed pathogens, with the exception of one species of roundworm, Syphacia obvelata, have been eliminated from the mice and rats in this colony by cesarian sectioning, foster-nursing, isolating, testing, slaughtering, and treating the animals. The quality of these animals is checked by killing sample animals and examining them for pathogens, and by testing all water bottles at least once a month for Pseudomonas aeruginosa and Salmonella

One animal caretaker and one technician are the only personnel regularly allowed in the breeding room. The cages used are chemically disinfected in addition to being sanitized with hot water as usual. Only autoclaved bedding is used. Water bottles, nipples, and stoppers are sanitized in the usual manner; however, special care is given to keep them separate from those used in other parts of the animal quarters. Specially handled, but unpasteurized food is used

Breeder Colony

The objective of this project is to develop a colony to supply breeders, free of all known pathogens, for the production colony.

By cesarian sectioning and foster-nursing, stocks of Sprague-Dawley rats, and CF No. 1 mice, free of all known pathogens, were developed from Lobund "germ-free" rats and mice. Similar stocks of C3H, C57, CBA, BALB/c, and A/Jax mice are also being developed.

The care and sanitation standards are the same as for the production colony with the exception that all water bottles are chemically disinfected as well as being sanitized as usual with hot water.

NUCLEAR STRUCTURES OBSERVED DURING DIVISION IN THE GIANT AMOEBAE

L. E. Roth

Dividing organisms of the giant amoebae, Pelomyxa carolinensis and P. illinoisensis, were selected from cultures that had been recently fed. They were fixed in 1% osmium tetroxide buffered to about pH 8.0 with 0.9% sodium chloride for 1 hr, dehydrated in progressive ethyl alcohols, and embedded in methacrylate. Further selection of stages was made by cutting a thick section for phase microscopy until the desired stages of division were located. This is possible since, in these organisms, several hundred nuclei are present and are almost always in close mitotic synchrony. Sections for electron microscopy were cut at 75 to 475 mμ settings of the microtome (Porter-Blum) and were usually overlaid with a methacrylate membrane(1) to reduce evaporation of methacrylate in the microscope (RCA EMU 3A).

The mitotic process in the nuclei is typical of metazoan nuclei with the major exceptions that centrioles are absent and that fragmentation of the nuclear envelope is delayed until the chromosomes are aligned on a metaphase plate. This latter characteristic may cause some confusion in the naming of stages, but will interfere little with our understanding of the events if stages are named according to the chromosome configuration. A light microscope study made by Kudo(2) on division in P. carolinensis has been helpful in this study.

The interphase nuclei are characterized by large, peripheral basophilic structures closely placed to the continuous nuclear envelope (Figure 2, E); these may be considered as nucleoli (Figure 2, N). The Feulgen-positive part of the resting nucleus is typically the central area, in which no distinctive structure is seen. The cytoplasm at this time has many granules that measure about 25 mμ in diameter; the nucleus lacks such granules.

Prophase is characterized by nucleolar changes: the nucleoli leave their peripheral location and disappear (Figure 3). By metaphase, chromosomes have condensed so that after osmium staining and fixation, they are denser to electrons than most other structures present (Figure 4, C); nucleoli are almost completely gone, the nuclear envelope is discontinuous (Figure 4, E) and the spindle fibrils (Figure 4, S) have formed inside the volume bounded by the nuclear envelope fragments. In addition to these rather typical metaphase events, granules measuring about 25 mμ in diameter that were previously present only in the cytoplasm are now seen in large numbers in close association with nuclear components (Figure 4b, G).

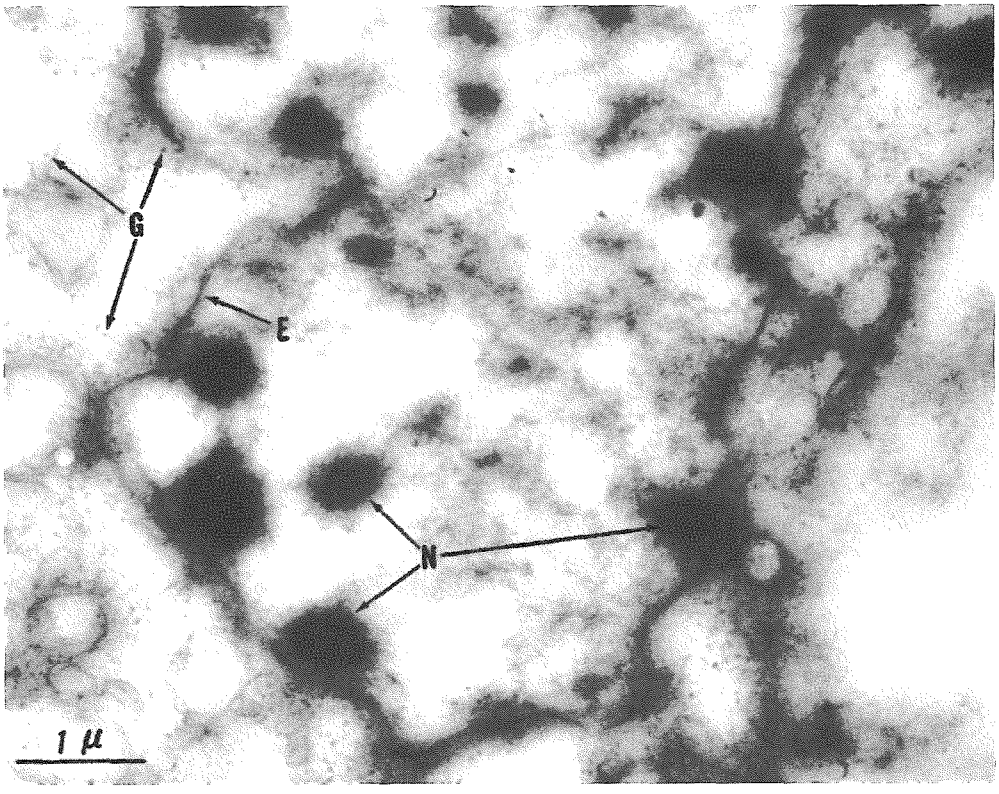

Figure 2. Interphase nucleus from Pelomyxa carolinensis

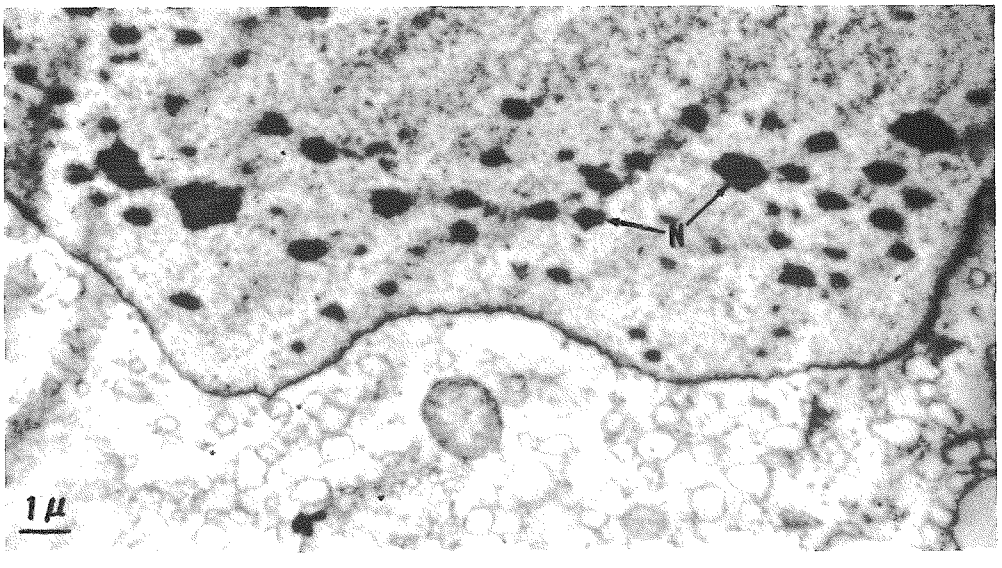

Figure 3. Prophase nucleus from Pelomyxa carolinensis

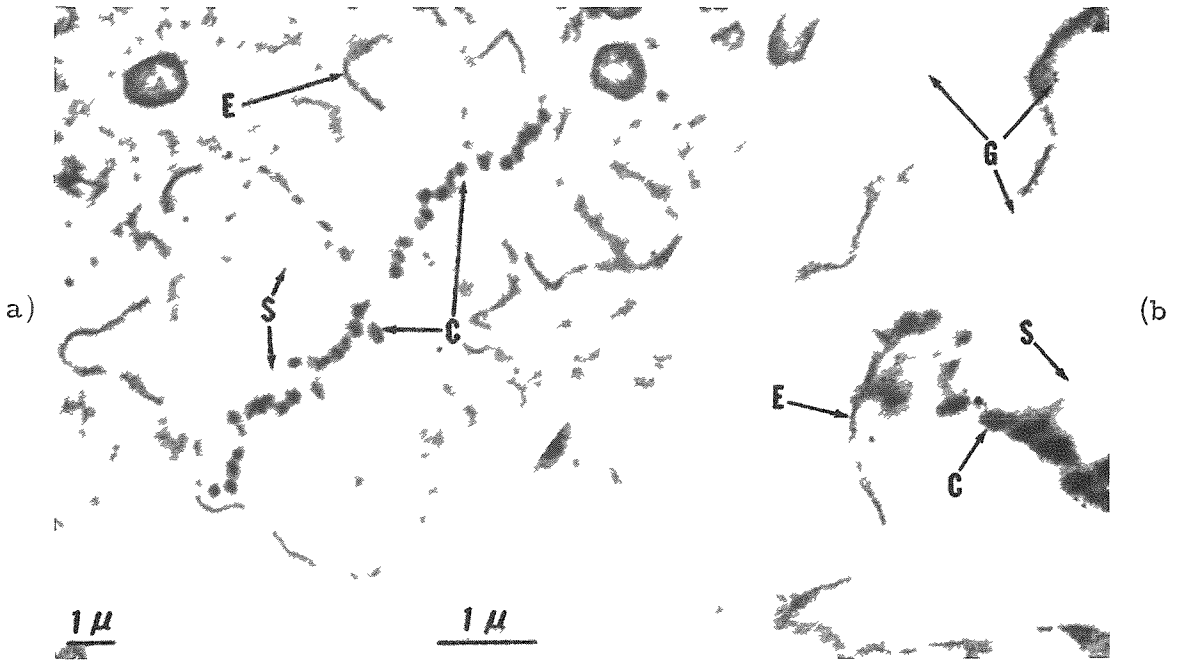

Figure 4. a. Survey of metaphase nucleus from Pelomyxa carolinensis,
b. Detail of metaphase nucleus from Pelomyxa carolinensis.

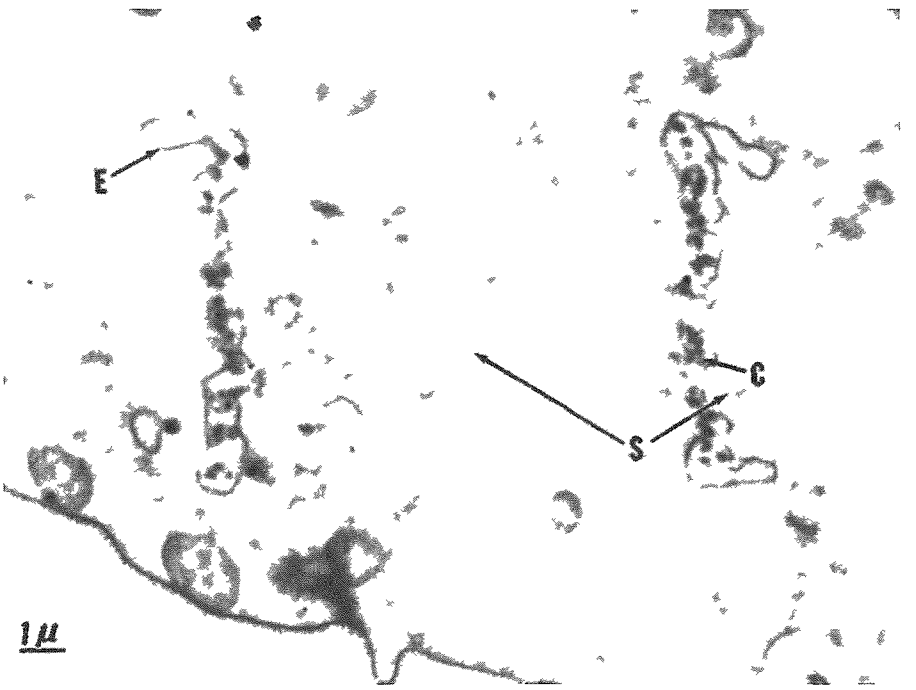

Figure 5. Late anaphase nucleus from Pelomyxa carolinensis

These particles are probably high in ribose nucleoprotein, so that their presence in this location at this stage may be highly significant. Two explanations are possible: they were formed from nuclear contents (perhaps from the RNA-rich nucleoli which have just disappeared) or were carried into the nuclear volume by a rather rapid and thorough mixing with the cytoplasm. Further study will be made of this phenomenon.

By late anaphase and telophase, the nuclear envelope has re-formed (Figure 5, E) except for openings through which spindle fibrils still pass; the spindle is now clearly outside the envelope and in the cytoplasm. No 25-mμ particles are present in the daughter nuclei in these stages.

Later telophase nuclei, in which young nucleoli are present, have also been observed; the envelope is continuous and the chromosomes no longer visible.

This study thus far has shown that giant amoeba, if properly selected, are valuable for the study of mitosis by electron microscopy. The division events are closely related to typical mitosis so that problems of condensation of chromosomes, formation of spindle fibrils, disappearance and reformation of nucleoli, and involvement of the nuclear envelope may be studied at the high resolutions attainable in modern electron microscopy.

References

1. Roth, L. E. The use of methacrylate membranes to protect thin sections from damage during electron microscopy. This report, p. 27

2. Kudo, R. R. Pelomyxa carolinensis Wilson II nuclear division and plasmotomy. J Morphol. 80 93-144 (1947).

OXIDATIVE PHOSPHORYLATION BY RAT-LIVER MITOCHONDRIA: NOTES ON THE INFLUENCE OF AGE, SEX, AND DIET

K. Surrey and S. A. Gordon

Introduction

During an investigation of mitochondrial phosphorylation, the commercial rodent diet in this laboratory was changed from Rockland* to Lab Blox.** The effect of this change on the phosphorylative potential of rat-liver mitochondria was determined for two groups of animals. Summarized below are observations on sex-paired siblings of Sprague-Dawley rats and their progeny that were maintained on the two rations from weaning to assay

Methods

Animals were stunned by a blow on the head and partially exsanguinated. The liver was excised and homogenized immediately in 8 times its weight of dispersion medium (0 25 M sucrose, 3 mM ethylenediaminetetraacetic acid, pH 7.2) The brei was filtered through nylon cloth and centrifuged 10 min at 600 x g. A capillary tube was used to remove the supernatant, which was centrifuged at 10,000 x g for 10 min. Again the supernatant was removed by aspiration. The residual mitochondria were resuspended in several ml dispersion medium, resedimented by centrifugation at 10,000 x g for 10 min, and taken up in 2.5 ml of dispersion medium per gram of liver originally dispersed. The suspensions contained ca 1 mg TCA-precipitable N per ml. At all stages the preparations were maintained below 4°C.

An aliquot of mitochondrial suspension was added to an incubation medium consisting of 18 μM $MgCl_2$, 3.5 μM ATP, 0.06 μM cytochrome c, 39 μM K_2HPO_4, 2 to 3 μc carrier-free $H_3P^{32}O_4$, 39 μM succinic acid, 18 μM ADP, and dispersion medium to make a final volume of 3.0 ml. All components of the medium were previously adjusted to pH 7.2. Upon addition of the mitochondria, the mixture was placed in the dark in a Dubnoff shaker maintained at 30°C. At several-minute intervals an aliquot (usually 0.1 ml) was removed for determination of inorganic and esterified phosphate by the tracer method of Ernster et al.(1) It will be shown elsewhere that ATP comprises essentially all of the phosphate ester produced during the incubation.

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*Rockland Mouse Diet, A. E. Staley Mfg. Co., Chicago

**Wayne Lab Blox, Allied Mills, Chicago

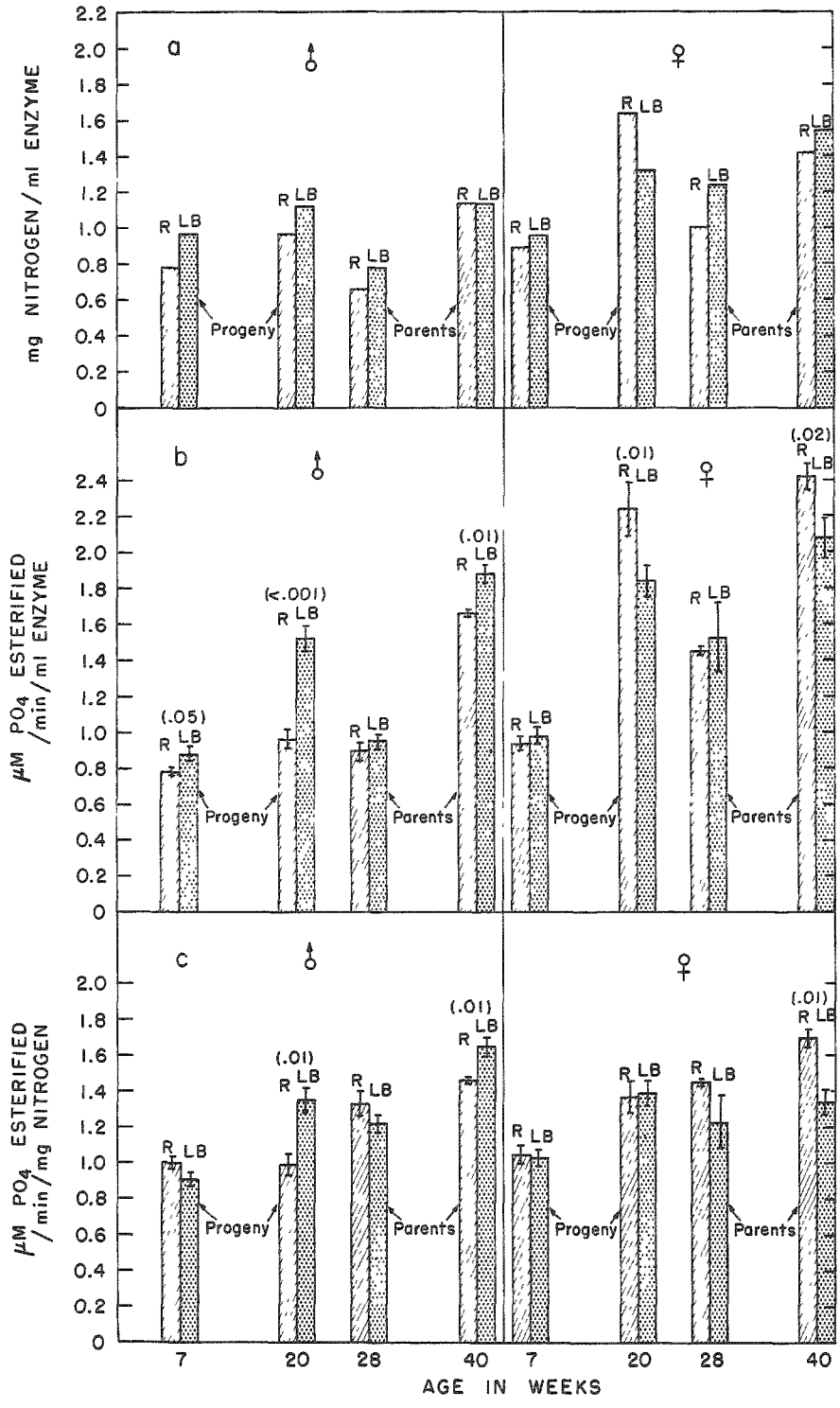


Figure 6. a, protein nitrogen; b, phosphorylative activity; c, specific activity of rat-liver mitochondria.

The numbers in parentheses indicate the percent level at which each difference is significant. R, Rockland and LB, Lab Blox diets.

Results and Discussion

The phosphorylative activities, protein nitrogen, and specific activities of the various suspensions are shown in the figures. The following inferences may be drawn.

1. Mitochondria of female animals have higher protein contents than do those of males. This difference is manifest under both diets (Figure 6a).

2. With one exception (20-week female progeny), the Lab Blox diet appears to result in slightly higher levels of mitochondrial protein (Figure 6a).

3. In part, these protein differences account for the higher phosphorylative activity in the females, both parents and progeny, at all ages under both diets (Figure 6b). This is also indicated by the decrease in difference when specific activities of both sexes are compared (Figure 6c).

4. Comparing the diets, Lab Blox appears to induce higher phosphorylative activity in the male progeny and parents, but only after 20 and 40 weeks of feeding, respectively (Figure 6b, left). On the other hand, Lab Blox induced lower activities in the females of corresponding age (Figure 6b, right). The higher mitochondrial activity in the males that were fed Lab Blox is also suggested by the specific activities in Figure 6c, left. However, the specific activities shown for the females in Figure 6c, right, indicate that only the 40-week female parents on Lab Blox exhibit significantly lower specific activity than those on Rockland.

These data indicate that both protein content and phosphorylative activity of rat-liver mitochondria were affected by the diet although the precise effect was dependent on sex and age.

Reference

1. Ernster, L., R. Zetterstrom, and O. Lindberg. A method for the determination of tracer phosphate on biological materials. Acta. Chem. Scand. 4 942-947 (1950).

PRECISION MEASUREMENT BEYOND THE LIMIT OF RESOLUTION WITH TRIANGULAR ARRAYS

Herbert E Kubitschek

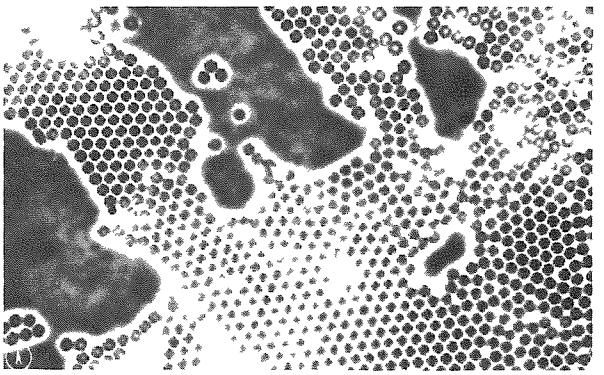
In a previous report[1] a rapid optical method was introduced for measurement of the mean diameters of monodisperse spherical particles, which may then be used as size standards in the microscopic range. Provided that the particles are found or arranged in two-dimensional triangular arrays, the method can be applied with extreme precision even to particles beyond the limit of resolution, and it also allows the determination of variation in particle size. The method consists simply of measuring the length of a line of particles in the array. Although to date only standard spherical monodisperse polystyrene particles have been sized, the method is applicable in principle to the study of spherical viruses and macromolecular crystals. This report is primarily concerned with the experimental determination of the correction factor for the true mean diameter.

The measurement of a row of particles in a triangular array gives a value slightly greater than the true mean diameter since adjacent particles of different diameters create small gaps in the array. Simple models indicate that the overestimate, $\overline{\Delta X}$, of the mean diameter, $\overline{X}$, should be directly proportional to the standard deviation σ of the mean particle diameter calculated from the measurements.

$$\overline{\Delta X} = k\sigma$$

To determine the coefficient k for this proportionality, model arrays were constructed of standard machinist's washers; one example is shown in Figure 7B. That these arrays are similar to those composed of 1-μ polystyrene latex particles may be seen by comparison with Figure 7A

Arrays of 100 washers of two sizes, and a further set with diameters selected to have a normal distribution (coefficient of variation, 2.36%) were measured in the standard arrangement of Figure 7B along each of the 20 rows of 10 washers. The observed mean incremental diameters $\overline{\Delta X}$ and their standard deviations σ are displayed in Figure 8 in per cent of the mean diameter. There measurements provide evidence for the linear relationship between $\overline{\Delta X}$ and σ when σ is less than 2% of the mean The same coefficient, $k = 0.4327$ (S.E.: 0.0087) can be fitted to both the 2-component and the normal distributions Knowledge of k allows its application as a correction in the calculation of the true mean diameter of the standard polystyrene particles in Table 3, as well as any other planar triangular array of spherical particle that may be examined. These diameters are slightly larger than those previously recorded,[1] presumably because the silicon replica grating used as an absolute standard in calibration had been stretched during its previous history. A more accurate calibration to within 0 1% has since been made against a machine screw calibrated in turn against standard machinist's blocks.



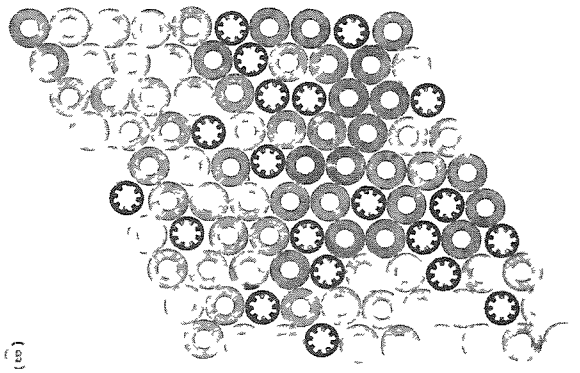


Figure 7

A: Triangular arrays of 1-μ polystyrene particles in oil, under phase contrast.
B: A model washer array.

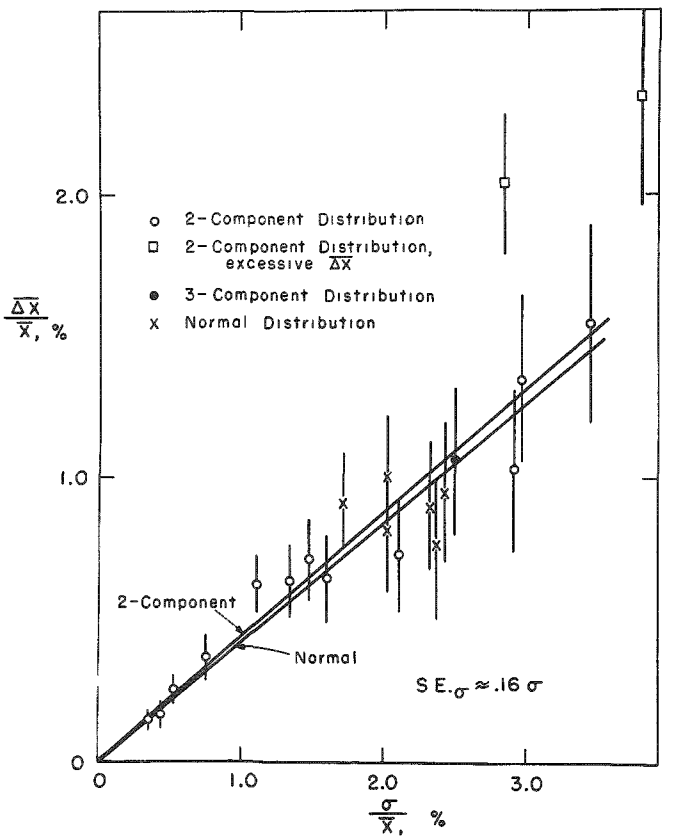


Figure 8

The observed linearity of the overestimate of the diameter, X, as a function of the standard deviation σ of washer diameters derived from measurements upon rows of washers. Linearity is lost for excessive $\overline{\Delta X}$.

TABLE 3

Mean and standard deviations of particle distributions of some monodisperse polystyrene latexes

Latex sample no.	Number of particles, n	Number of rows, N	Mean measured diameter, $\overline{X}$, μ	S.E. of $\overline{X}$, μ	$\frac{\sigma}{X}$, %	Corrected diameter, μ
15N-8	105	17	0.518	0.0065	3.83	0.510
LS-066-A	640	39	0.799	0.0013	0.78	0.795
LS-067-A	617	52	1.157	0.0015	0.68	1.154
L-3830-1	78	8	1.708	0.0043	0.26	1.702
L-3830-19-B	57	9	3.222	0.0069	0.39	3.217

The variance of particle diameters obtained from the measurements upon the array is expected to provide a direct estimate of the true variance of particle diameter when the variance of the distribution of gaps is much smaller than these, and may therefore be neglected. This was tested for the washer arrays by comparison of the standard deviation σ from the 20 measurements of rows in each array with the true standard deviation σ_{tr} obtained from measurements of individual washer diameters. As shown in Figure 9 these standard deviations are essentially the same when the overestimated mean is within 1% of the true mean. This is the case for most of the polystyrene particles. The corrected mean diameters are also shown in Table 3.

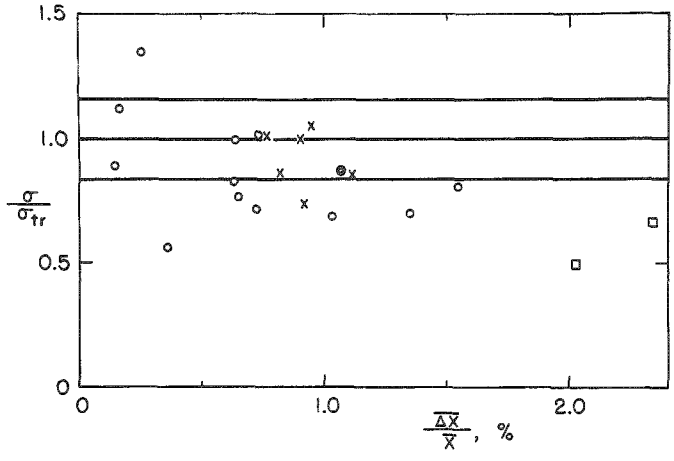


Figure 9. Agreement between the measured standard deviation σ and the true standard deviation σ_{tr} for washer arrays. The apparent differences at larger $\frac{\Delta X}{X}$ may be fallacious. Symbols same as in Figure 8.

Reference

1. Kubitschek, H. E., and J. F. Thomson. Absolute measurements of spherical particles of some polystyrene latexes. Biological and Medical Research Division Semiannual Report, Argonne National Laboratory. ANL-5916, (1958) pp. 45-46.

THE ROLE OF TRANSCARBOXYLATION IN PROPIONIC ACID SYNTHESIS

Robert W. Swick and Harland G. Wood

It has generally been considered that propionate is formed in propionibacteria through conversion of pyruvate to oxalacetate by fixation of CO_2, reduction of oxalacetate to succinate, then esterification with coenzyme A (CoA) and decarboxylation to propionyl CoA and CO_2. This conversion should involve the turnover of 1 mole of CO_2 per mole of propionate formed. However, in isotope experiments designed to measure these reactions, the observed turnover of CO_2 was much less than 1, and it was concluded that a C_1 unit other than free CO_2 was a product of the cleavage.[1] Phares, et al [2] also obtained evidence suggesting that a C_1 unit was formed during the decarboxylation of succinate: isotopic propionyl CoA was incorporated into succinyl CoA by extracts of Propionibacterium pentosaceum more rapidly than labeled CO_2

Succinate has also been shown to be involved in the catabolism of propionate in animal tissues However, Flavin and Ochoa[3] demonstrated that the primary product of the carboxylation of propionyl CoA was not succinyl CoA but methylmalonyl CoA, and that succinyl CoA was only formed in a subsequent reaction from methylmalonyl CoA.

The present studies with cell-free extracts of P. shermanii have demonstrated that the conversion of pyruvate to propionate can occur without the formation of free CO_2 and that methylmalonyl CoA is an intermediate in the conversion [4] The results may be summarized by the following set of equations·

Succinyl CoA	$\rightleftharpoons$	Methylmalonyl CoA	(1)
E* + Methylmalonyl CoA	$\rightleftharpoons$	Propionyl CoA + E-C_1	(2)
E-C_1 + Pyruvate	$\rightleftharpoons$	Oxalacetate + E	(3)
Oxalacetate + 4H	$\rightleftharpoons$	Succinate	(4)
Succinate + Propionyl CoA	$\rightleftharpoons$	Succinyl CoA + Propionate	(5)
Net· Pyruvate + 4H	$\rightleftharpoons$	Propionate	(6)

Reaction (1) involves an isomerization and reactions (2) and (3) involve a transcarboxylation, both of which are novel biochemical reactions.

*Enzyme

Methods

P. shermanii (52W) was grown in glucose-yeast extract medium and harvested by centrifugation, washed, lyophilyzed, and stored at -10°. The dry cells were suspended in neutral, isotonic KCl, mixed with glass beads and shaken repeatedly in a Nossal shaker. The mixture was centrifuged to remove the beads and cell debris, and the supernatant solution was passed through Dowex 1-Cl^- to remove the nucleotides. The precipitate obtained by the addition of ammonium sulfate to 90% of saturation was dissolved in phosphate buffer and dialyzed. The extracts were stored at -12° and remained active for a month or longer.

The coenzyme A derivatives were prepared either from the appropriate acid anhydride or from a mixed anhydride. Propionate-C^{14} and succinate-C^{14} were purchased, oxalacetate-4-C^{14} was prepared enzymatically, and methylmalonic acid was prepared chemically.

In order to measure the incorporation of propionyl-C^{14} CoA into the mixed dicarboxylic acids, the appropriate compounds were incubated in phosphate buffer at pH 7 at 30°. The reaction was stopped by the addition of alkali, and the CoA derivates were hydrolyzed in a boiling water bath. The reaction mixture was made acid, mixed with Celite and packed into a short column. The organic acids were eluted with ether, transferred back to alkali and plated for measurement of the total radioactivity. The planchets were made acid and re-evaporated, whereupon propionic acid distilled off leaving only the nonvolatile dicarboxylic acids to be counted.

When it was desired to isolate propionic, methylmalonic and succinic acids individually, carrier was added, and the mixture was chromatographed on Celite, with chloroform containing various amounts of n-butanol as the eluting solvent. The compounds were located by titration and plated as before.

Results

It has been observed with resting cells and with extracts of propionibacteria that propionate is equilibrated with succinate. The present extracts bring about a similar exchange when propionyl CoA and succinyl CoA are the substrates: some 30% of the radioactivity of the propionyl-C^{14} CoA added was isolated as C^{14}-dicarboxylic acids. When the free acids were used, it was also necessary to add CoA and ATP The net synthesis of C^{14}-dicarboxylic acids occurred with CO_2, ATP, CoA and propionate-C^{14}, presumably by conversion of the CO_2 to the active C_1. When extracts were prepared from lyophilized cells which had been stored for several months, there was no activity with CO_2 while the activity with succinate as the source of a C_1 was undiminished. This lends support to the postulate that the transcarboxylation reaction involves a C_1 unit which is not CO_2.

Reaction (2) involves the formation of the C_1 unit from methylmalonyl CoA. It was of interest to learn whether other compounds which were known to yield CO_2 during their catabolism would also form a C_1 unit which would be transferred to propionyl CoA. It is seen in Table 4 that in the presence of succinate, ATP, and CoA there was conversion of propionate-C^{14} to dicarboxylic acids; however, oxalacetate was much more effective than succinate. These results are in accord with reaction (3) in which a C_1 would be formed from oxalacetate and transferred to propionyl CoA to form methylmalonyl CoA, reaction (2). Other experiments in Table 4 indicate that oxalacetate probably was not converted to succinate prior to transcarboxylation with propionyl CoA. Nor is it likely that oxalacetate was first decarboxylated and the free CO_2 fixed to propionyl CoA since the yield of dicarboxylic acids was only about $\frac{1}{10}$ as great with bicarbonate as with oxalacetate. Furthermore, other extracts, which were prepared from aged cells and which would not fix CO_2, readily utilized oxalacetate for transcarboxylation. Formaldehyde, formate, pyruvate, and 6-phosphogluconate were also inactive in the transcarboxylation.

TABLE 4

The carboxylation of propionyl-C^{14} CoA by various compounds

Additions*	C^{14} in dicarboxylic acids formed,** μmoles	Additions*	C^{14} in dicarboxylic acids formed,** μmoles
Succinate	0.036	Fumarate	0.006
Oxalacetate	0.117	Isocitrate	0.002
Oxalacetate + malonate	0.120	α-Ketoglutarate	0.003
Malate	0.016	$KHCO_3$***	0.014

* The system contained (in μmoles): potassium phosphate, pH 7, 50; propionate-C^{14}, 0.3; CoA, 1; ATP, 1; enzyme with 4 mg of protein; and where indicated, succinate, oxalacetate, isocitrate, α-ketoglutarate, malate, fumarate, 1; malonate, $KHCO_3$, 50. Final volume, 0.5 ml; incubated 30 min at 30°.

** Presumably a mixture of succinic and methylmalonic acids. Calculated in μmoles from the total nonvolatile radioactivity and the specific activity of the propionate.

*** 3 μmoles of ATP were present in this case.

The final products of the transcarboxylation reaction between propionyl CoA and oxalacetate are succinyl CoA and pyruvate. Succinyl CoA was demonstrated by the preparation and chromatographic separation of radioactive succinhydroxamic acid. The formation of pyruvate was indicated by an enhanced oxidation of DPNH with lactic dehydrogenase when propionyl CoA was added to control vessels containing oxalacetate.

The formation of oxalacetate from pyruvate and a C_1 unit was investigated using succinyl-1, 4-C^{14} CoA as the C_1 donor, i.e., via

methylmalonyl CoA. The oxalacetate formed was separated from residual pyruvate by partition chromatography and degraded. All of the radioactivity in the oxalacetate carboxyl groups was present in the β-carboxyl moiety. Had oxalacetate arisen from succinate by oxidation, radioactivity would have been present in both carboxyl groups. In addition to oxalacetate, propionyl-C^{14} CoA and $C^{14}O_2$ were formed in the above reaction. There was some formation of $C^{14}O_2$ in the absence of added pyruvate, presumably by conversion of the bound C_1 to CO_2.

Flavin and Ochoa[3] showed in mammalian tissue preparations that methylmalonyl CoA is the primary product of the carboxylation of propionyl CoA by CO_2. This compound has now been shown to be the initial product of the transcarboxylation reaction in propionibacteria as well. Propionyl CoA and oxalacetate-4-C^{14} were incubated with the bacterial extract for various periods, the reaction halted, and carrier methylmalonate and succinate were added to the reaction mixture and re-isolated chromatographically. Both labeled methylmalonate and succinate were present: maximum methylmalonate formation occurred within 5 minutes and thereafter decreased, while succinate formation, which initially lagged behind methylmalonate formation, increased until succinate composed 81% of the mixture (Table 5).

TABLE 5

Relative yields with time of methylmalonate and succinate formed from propionyl CoA and oxalacetate-4-C^{14}

Duration of incubation, min	Methylmalonate formed, μ moles*	Succinate formed, μ moles*	Total acids formed, μ moles
1	0.139	0.032	0.171
5	0.170	0.071	0.241
20	0.125	0.125	0.250
40	0.058	0.247	0.305

* Calculated on the basis of the total radioactivity recovered and from the specific activity of the oxalacetate.

The complete system contained (in μmoles): potassium phosphate, pH 7, 50; propionyl CoA, 0.4; oxalacetate-4-C^{14}, 1; and enzyme with 4 mg of protein. Final volume, 0.5 ml; incubated as indicated at 30°.

When the extracts and pyruvate and either methylmalonyl CoA or succinyl CoA were incubated for 1 minute and the formation of oxalacetate measured with malic dehydrogenase, there was more than 10 times as much oxalacetate formed from methylmalonyl CoA as from succinyl CoA during the brief reaction period (Table 6). Therefore, it seems reasonable to conclude that the compound which is the immediate C_1 donor is methylmalonyl CoA rather than succinyl CoA. It also seems clear that the

rate-limiting reaction is the conversion of succinyl CoA to methylmalonyl CoA since upon longer incubation the yield of oxalacetate from succinyl CoA was similar to that obtained from methylmalonyl CoA.

TABLE 6

Formation of oxalacetate from pyruvate and methylmalonyl CoA or succinyl CoA

Substrate*	Oxalacetate formed,** μmoles
Pyruvate + methylmalonyl CoA (0.65 μmole)***	0.325
Pyruvate + methylmalonyl CoA (0.4 μmole)***	0.203
Pyruvate + succinyl CoA (0.58 μmole)	0.022
Pyruvate + succinyl CoA (0.58 μmole)	0.020

*System contained potassium phosphate, pH 7, 50 μmoles; pyruvate, 10 μmoles; methylmalonyl CoA or succinyl CoA as indicated and enzyme with 2 mg of protein. Final volume, 0.5 ml; incubated 1 min at 30°.

**Reaction was stopped by the addition of TCA to give a concentration of 2 per cent and the preparation was centrifuged and neutralized. Oxalacetate was assayed in an aliquot by observing the change in optical density at 340 mμ after the addition of 0.4 μmole of DPNH and malic dehydrogenase.

***As active isomer.

Delwiche[5] has shown that resting cells of P. pentosaceum grown on a medium low in biotin decarboxylate succinate slowly and that the addition of biotin stimulates this decarboxylation. Therefore, experiments were performed to ascertain the effect of avidin, an inhibitor of biotin activity, on reactions (2) and (3). It is seen in Table 7 that the addition of 200 μg of avidin almost completely inhibited the transfer of a carboxyl moiety from oxalacetate or succinate (via methylmalonyl CoA). This inhibition was prevented by the addition of 0.08 μmole of biotin simultaneously with the avidin.

Assay of the enzymes involved in the esterification of propionate, succinate, and methylmalonate showed that there was neglible formation of the CoA derivatives of succinate and methylmalonate while propionate was readily esterified.

The transfer of CoA between the dicarboxylic acids and propionate was also studied. It is seen in Table 8 that there is considerable incorporation of C^{14} from propionyl-C^{14} CoA into the dicarboxylic acids with either methylmalonyl CoA or succinyl CoA. With propionyl CoA and methylmalonate the incorporation of C^{14} is neglible but with succinate it is comparable to that with succinyl CoA. The succinate apparently may be activated by a transfer of CoA (which is reversible) from propionyl CoA as

TABLE 7

Effect of avidin and biotin on the carboxylation of propionyl-C^{14} CoA by oxalacetate or succinate

Additions*	C^{14}-dicarboxylic acids formed in the presence of	
	Oxalacetate,** μmoles	Succinate,** μmoles
None	0.129	0.054
Avidin	0.002	0.002
Avidin + biotin	0.140	
Biotin	0.122	

* The system contained (in μmoles): potassium phosphate, pH 7, 50; propionyl-C^{14} CoA, 0.3; oxalacetate or succinate, 1; enzyme containing 4 mg of protein; and where indicated, biotin, 0.08, avidin, 200 μg. Final volume, 0.5 ml; incubated 30 min at 30°.

** Calculated as described in footnote to Table 4.

previously reported, but there appears to be little transfer of CoA between propionyl CoA and methylmalonate. Methylmalonate likewise was not activated by ATP plus CoA. These results also demonstrate that methylmalonate must be esterified with CoA before it can be converted to succinate.

TABLE 8

CoA transfer as measured by the carboxylation of propionyl-C^{14} CoA

Substrate*	C^{14}-dicarboxylic acids formed,** μmoles
Propionyl-C^{14} CoA + methylmalonate	0.001
Propionyl-C^{14} CoA + methylmalonate + ATP + CoA	0.003
Propionyl-C^{14} CoA + methylmalonyl CoA	0.084
Propionyl-C^{14} CoA + succinate	0.060
Propionate-C^{14} + succinyl CoA	0.072

* The system contained (in μmoles): potassium phosphate, pH 7, 50; propionyl-C^{14} CoA or propionate-C^{14}, 0.3; methylmalonate, succinate, succinyl CoA, 1; methylmalonyl CoA (active isomer), 0.5; CoA, 1; ATP, 3; enzyme containing 4 mg of protein. Final volume, 0.5 ml; incubated 30 min at 30°.

** Calculated as described in footnote of Table 4.

To test the specificity of the transcarboxylation reaction, a few preliminary experiments were performed with the extracts, using other acceptors and donors. Oxalacetate and acetyl CoA give rise to malonyl CoA, while

oxalacetate and butyryl CoA formed ethylmalonyl CoA. In addition malonyl CoA and ethylmalonyl CoA will transcarboxylate with propionyl CoA, presumably yielding methylmalonyl CoA.

Discussion

In animal tissues the carboxylation of propionyl CoA is a one-step reaction involving CO_2 and ATP[6] and is inhibited by avidin while in propionibacteria this reaction involves a transcarboxylation which also can be inhibited by avidin but which requires no ATP. (The fixation of CO_2 into methylmalonyl CoA by propionibacteria, however, does require ATP.) It has been shown in animal tissues that the cleavage of methymalonyl CoA to CO_2 and propionyl CoA is accompanied by the regeneration of ATP;[6] nevertheless, since transcarboxylation does not require the activation of CO_2 and avoids the formation of free CO_2, it may provide more favorable kinetics for the transfer of a carboxyl group from one compound to another.

Recently, evidence has been presented for the formation of a CO_2-biotin-enzyme complex as an intermediate in the fixation of CO_2.[7] The inhibition of transcarboxylation by avidin indicates that biotin functions as a cofactor in the latter reaction as well; therefore, transcarboxylation may be mediated by a similar complex. While the formation of the complex from CO_2 requires ATP, it appears from the present results that a C_1-enzyme complex can also arise without participation of ATP by transfer of a C_1 from the proper donor compound. The structural requirements for a donor compound, apparently, are that it have a carbonyl group beta to the carboxyl group being donated and adjacent either to another carboxyl group, as in oxalacetate, or to coenzyme A, as in methylmalonyl CoA.

Ammonium sulfate fractions of ox liver, dog skeletal muscle, dog heart muscle, and a preparation of rat liver mitochondria disrupted by sonic treatment were tested for activity in a cursory quest for the transcarboxylation reaction in animal tissues. Although all were able to fix CO_2 with propionyl CoA and ATP, only the dog skeletal muscle preparation catalyzed the formation of dicarboxylic acids from propionyl CoA and oxalacetate. The results suggest that transcarboxylation is not confined to bacteria

The mechanism of the conversion of methylmalonyl CoA to succinyl CoA is uncertain at this time and will be explored in a later report.

The earlier failure to obtain the expected turnover of CO_2 during propionate synthesis *in vivo* with *P. shermanii* now has a demonstrable explanation The unesterified carboxyl group of methylmalonyl CoA need not be released as free CO_2 but may be transferred to pyruvate with the formation of oxalacetate which can be reduced to succinate, etc. Thus

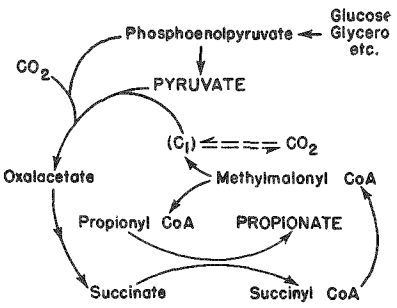


Figure 10

only catalytic amounts of oxalacetate need be produced by fixation of CO_2. Likewise, coenzyme A may be recycled and only catalytic amounts of the acyl derivatives need be formed by de novo synthesis requiring the expenditure of ATP. The scheme shown in Figure 10 is consistent with the results of this study and is proposed as the pathway of propionate formation from pyruvate in propionibacteria.

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THE USE OF METHACRYLATE MEMBRANES TO PROTECT THIN SECTIONS FROM DAMAGE DURING ELECTRON MICROSCOPY

L. E. Roth

It has been shown that thin sections (50-300 mμ thick) of methacrylate-embedded tissues undergo a succession of changes during initial electron bombardment. The result of these changes has been shown[1] to be a loss of about one-half of the embedding methacrylate in the first few seconds of electron bombardment; the percentage may be higher or lower dependent upon the microscope characteristics and manner of beam manipulation by the microscopist. This phenomenon has been essentially ignored by most microscopists although minor efforts to minimize the difficulty have been made. Watson,[2] however, has proposed that sections should be "sandwiched" between carbon and Formvar membranes and claimed such a great reduction of the effect that it is no longer a significant problem. This method, although an improvement, thickens the specimen and reduces contrast so that a loss of resolution may also result.

A solution to the problem would be to overcoat the section with a substance which is removed by electron bombardment adjusting the thickness of material applied to the loss expected; such a material is methacrylate, and membranes can be made of the same composition as used for embedding tissue. Methacrylate membranes can be formed on a water surface from a 2%, well-dried solution of polymerized methacrylate in methyl methacrylate monomer. A "sandwich" with the section placed between a carbon and methacrylate membrane is formed by one of several possible methods. When bombarded by electrons in the microscope, the methacrylate membrane sublimes or evaporates but not until the methacrylate which is present in the section has stabilized (probably by chemical change) The result is a section and supporting film of the thinnest possible dimension The technique, therefore, allows photographs of sections which have neither the disadvantage of methacrylate loss nor the loss of contrast of conventionally "sandwiched" sections.

Sections thus prepared, when observed in the microscope, show very sharp membrane contours and require less effort to focus for photography. The micrographs included in another presentation in this report[3] were made by this technique

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THE ENVELOPE OF THE FAT CELL IN THE ELECTRON MICROSCOPE

F. Wassermann and T. F. McDonald*

The histologists believe that the fat cell is furnished with a membrane which is of functional significance for its specific cellular activity and, also according to Wassermann's experimental studies[1] for the uptake and the release of water. Light microscopic observations of the fat cell wall in sections of adipose tissue did not determine whether it included a true cell membrane, that is a distinct cytoplasmic structure at the surface of the cell, covered by a layer of the surrounding connective tissue, or whether it was made up only of connective tissue.[1] It was the purpose of the present electron microscopic investigation to settle this question.

Material and Methods

Small pieces of adipose tissue from the fat bodies at the testis and in the inguinal region of Sprague-Dawley rats were fixed in the osmic acid-dichromate mixture of Dalton.[2] The tissue was embedded in methylacrylate and sectioned with the Porter-Blum microtome. The observations were made with the EMU A2 and A3 RCA electron microscopes. In addition to animals which were kept on the ordinary laboratory diet ad libitum we also used rats that had suffered severe loss of body weight by starvation and animals that after periods of starvation were given food rich in fat (in the form of bacon) and were killed at intervals between one-half and 24 hr after refeeding. The material from starved rats was supplemented by adipose tissue from guinea pigs which were emaciated in the course of experimental scorbutus. The fat cells of such tissues were electron microscopically identical with those of starved rats.

Observations

Part of a cell of the inguinal fat body from a one-day-old rat is shown in Figure 11. The cytoplasm, which contains numerous mitochondria, covers a slightly osmiophilic material that appears to be characteristic of the beginning of the fat-producing activity of the cell. At the surface of the cell one notices two parallel lines separated by a narrow space. The lines are of equal width, about 80 A, and the space between them measures about 100 A. The regular occurrence of a double contour of the fat cells makes it certain that the lines represent two lamellae. Depending on the angle of the lamellae to the plane of sectioning, the two lines are more or less distinct in the electron micrographs. That they are discontinuous in the pictures is therefore no reason to doubt the continuity of the lamellae themselves.

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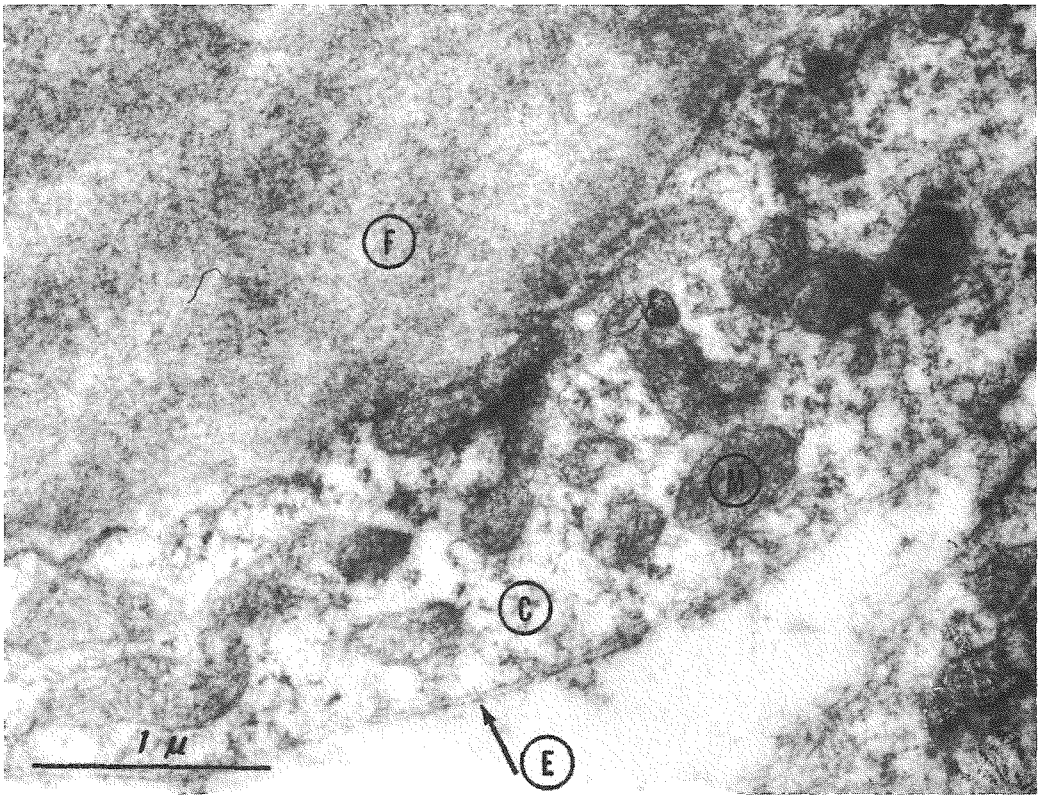

Figure 11. Part of the surface region of a fat cell from the inguinal fat body of a one-day-old rat. The intracellular fat droplet (F) is covered by a cytoplasm (C) with mitochondria (M). The cell border is marked by a double line. It represents the outer and the inner lamellae plus the interlamellar space of the envelope (E).

The double contour of the fat cell is very similar to the double membranes frequently described by electron microscopists as enveloping nuclei or mitochondria. For reasons which will become evident later on we do not call the two lamellae a double membrane but prefer to describe the structures at the surface of the fat cell as an envelope composed of two lamellae and an interlamellar space.

Another example of the appearance of the envelope of a fat cell from a sixty-day-old rat is shown in Figure 12 at a higher magnification than that of Figure 11.

Figure 13 shows the surface of a fat cell of an adult rat in the region where the nucleus lies between the blackened fat droplet and a thin layer of cytoplasm which separates the double nuclear membrane from the similar double contour of the envelope. This picture serves to demonstrate the inner lamella of the envelope as a structure which can be distinguished from the cytoplasm proper even in the large fat cell. In the light microscope the cytoplasmic shell around the fat droplet and the nucleus is reduced to an indistinguishable layer.

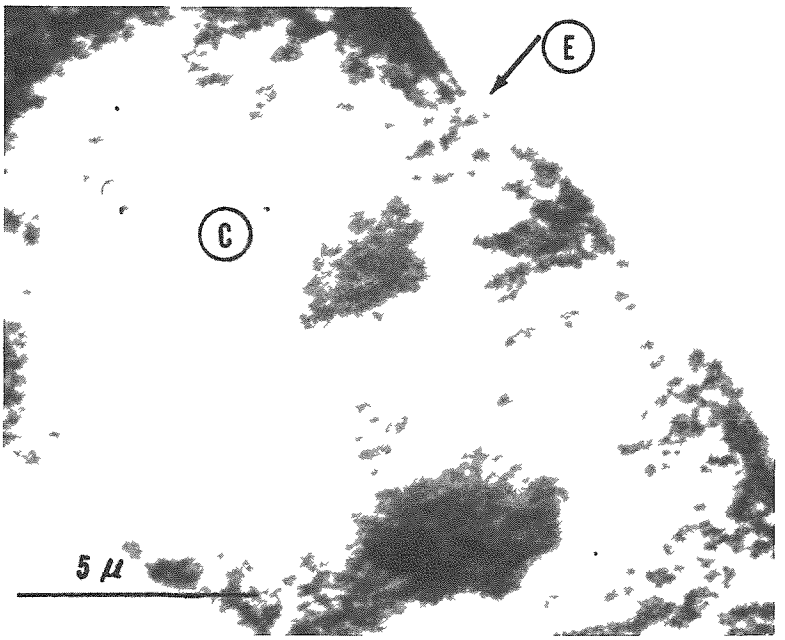

Figure 12. The cytoplasm (C) of the fat cell from an adult rat. At (E) the two lamella of the envelope are each about 80 A wide and are separated by the interlamellar space of 80 to 100 A in width.

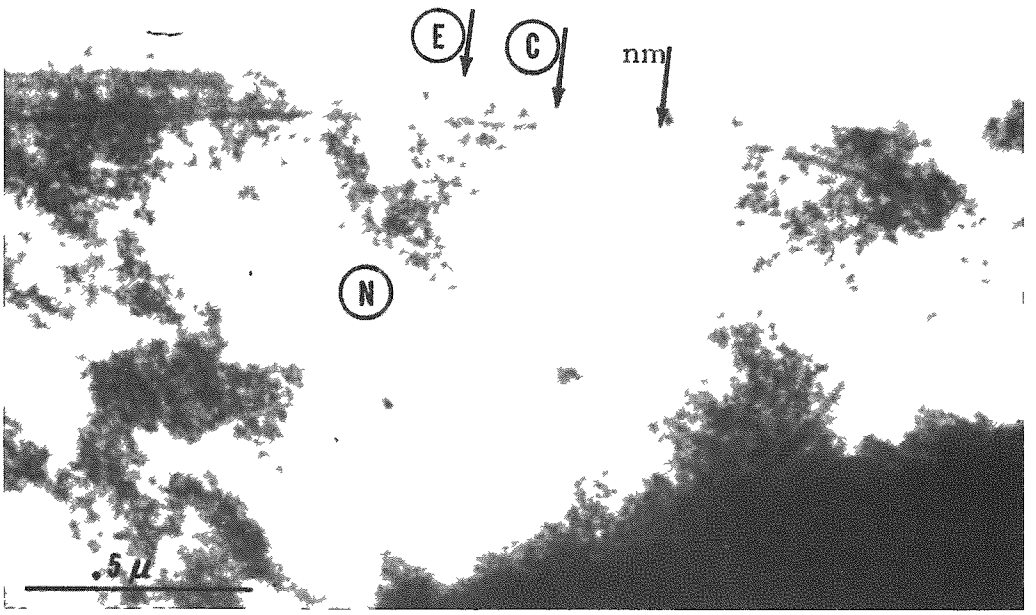

Figure 13. Part of a fat cell with the osmium blackened fat droplet and the nucleus (N). Notice the double line of the nuclear membrane (nm), the double line of the envelope and the narrow layer of cytoplasm (C).

According to our findings the aspect of the cell surface changes with the phase activity in which the cell was caught at the moment of fixation. In the electron microscope one occasionally finds fat cells that show an accumulation of a great mass of osmiophilic granules under the surface within the cytoplasm on top of the main fat droplet (Figure 14). The cell was from an 80-day-old rat kept on the usual diet. Such pictures are not surprising since previous histological and experimental studies led to the conclusion that the fat bodies are not inert store houses of reserve material but rather are the organs of peripheral fat metabolism,[3] and are continually engaged in synthesizing and releasing lipids.[4] This concept was verified a few years later by the classic work of Schoenheimer and Rittenberg[5,6] who demonstrated the continual metabolic turnover of adipose tissue by the use of deuterium-labeled compounds.

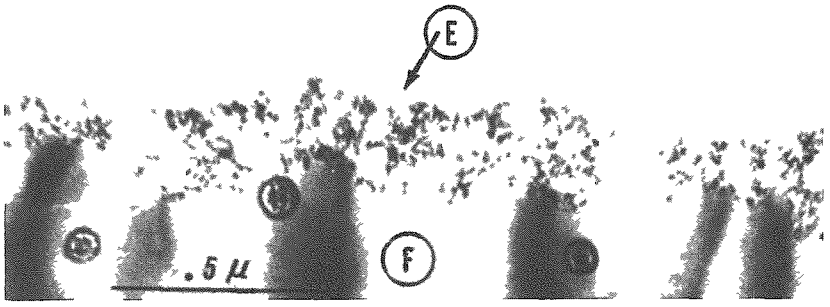

Figure 14
Part of the surface of a fat cell from an adult rat. Notice the osmiophilic granules between the fat droplet (F) and the envelope (E) of which only the outer lamella is clearly seen.

The significance of pictures such as that of Figure 14 is confirmed by producing the same situation experimentally in animals which were refed after starvation. The osmiophilic granules in Figure 15 which are very similar to those in Figure 14, most likely represent lipid material resynthesized by the cell after the components were taken up through the outer lamella of the envelope. It was of special interest to see that the outer lamella and the interlamellar space remain distinct under variable functional conditions of the cell. Since the inner lamella encloses the same granules as the rest of the cytoplasm in Figures 14 and 15, one can no longer distinguish it as a separate structure.

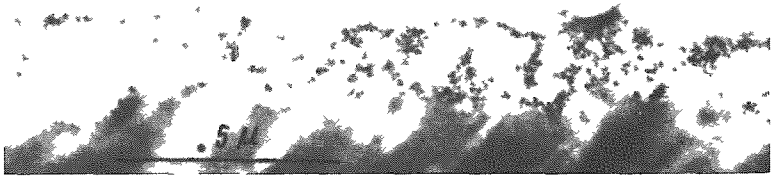

Figure 15. Similar to Figure 14. The outer lamella of the envelope is distinct, the inner is obscured due to the dark granules. The cytoplasm is filled with granules. This cell was taken from an animal which after starvation with a loss of 30 per cent body weight was refed 30 min before fixation of the tissue.

Functional changes in the envelope were found in the fat cells from starving animals (Figure 16). It is known from light microscope observations that the shrinking fat cell while releasing most of its stored material changes from a spherical body into an irregularly shaped one with numerous processes.(1) The electron microscope reveals details in the configuration of the cell surface in the form of numerous protrusions along the processes. The envelope itself can be found unchanged in some places (arrows in Figure 16), but over most of the surface the outer lamella, removed from the still distinct inner lamella, encloses pouches and sometimes branching tubules that are extensions of the interlamellar space. The possible extent of the enlargement of the interlamellar space during the release of the fat can be seen in Figure 17. Both this figure and Figure 16 show the connective tissue in the vicinity of the fat cells.

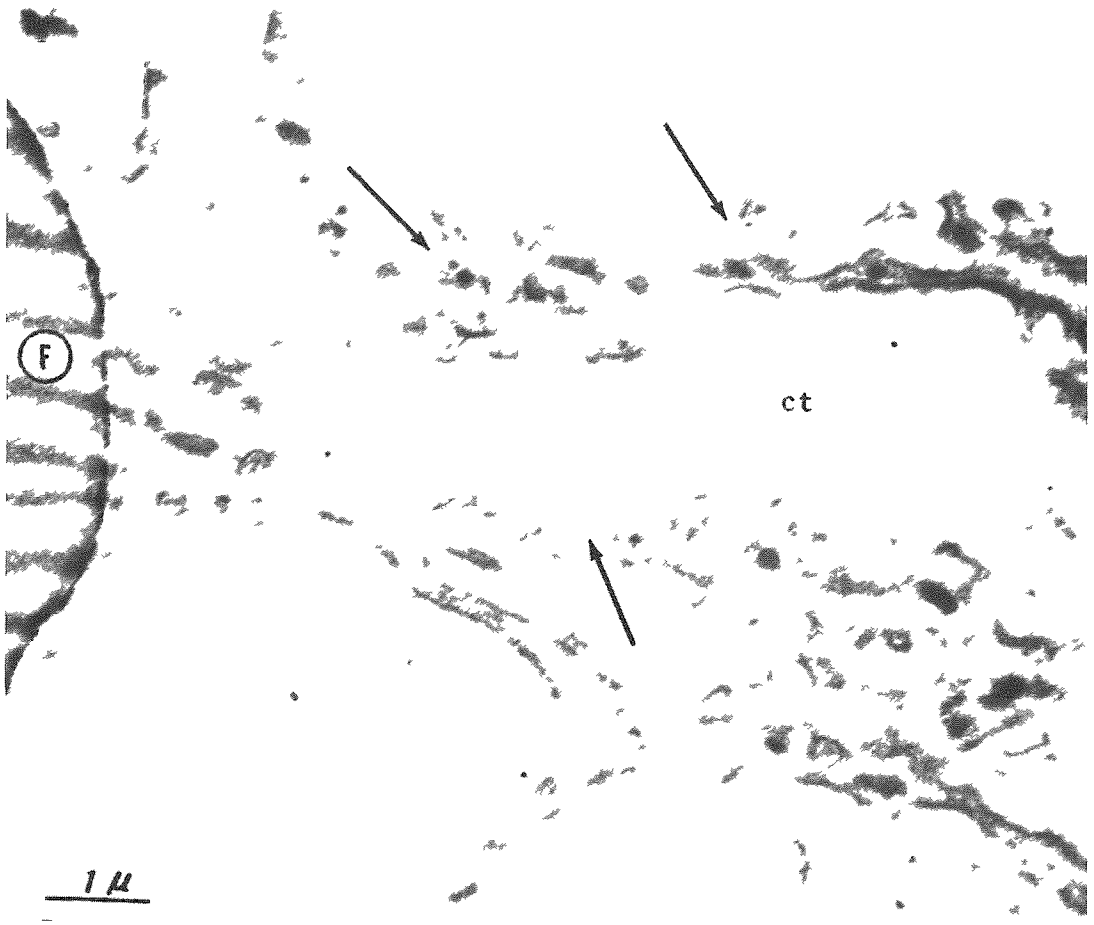

Figure 16. Part of the surface of a fat cell of the testicular fat body of a starved adult rat. Notice the fat droplet (F) at the left, the cytoplasm and the mitochondria and the extensions of the cell body with the protrusions at their surface, (ct) indicates connective tissue. The arrows point to places where the envelope is in the same state as in Figures 11, 12, and 13. Otherwise the outer lamella is removed from the inner, enclosing vesicular and tubular extensions of the interlamellar space.

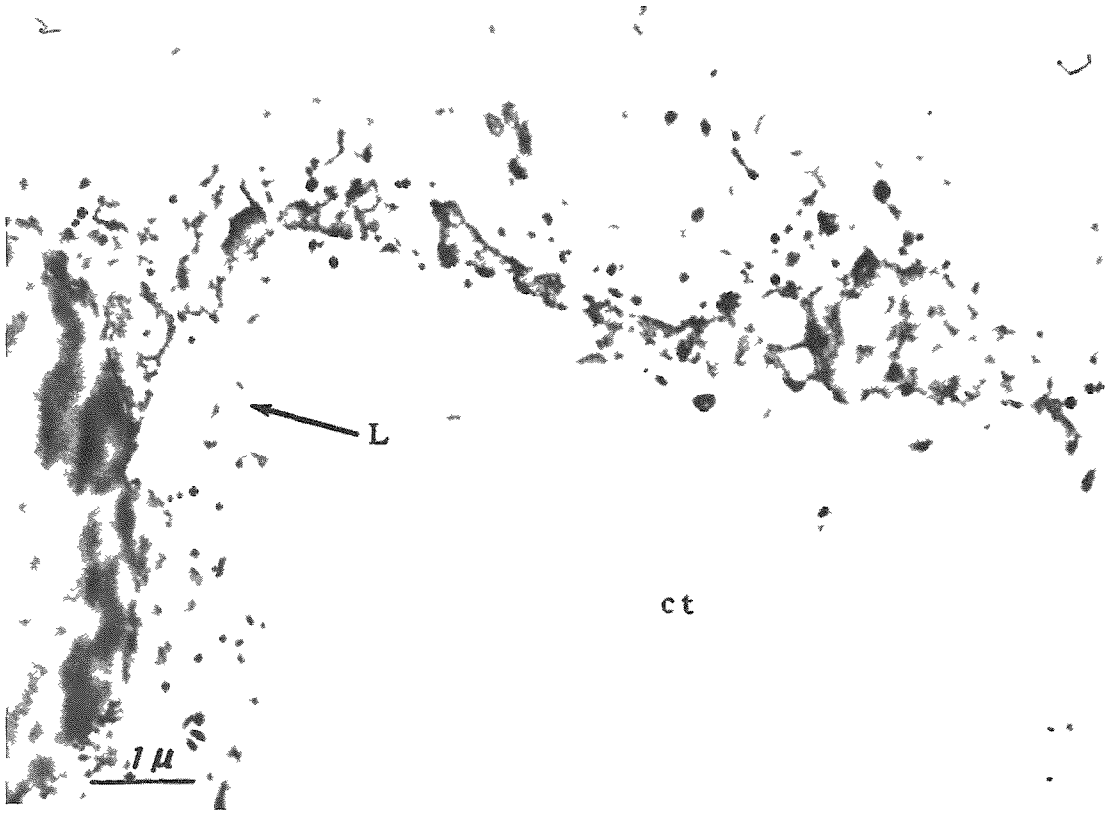

Figure 17. Part of the surface of a fat cell from a starved rat where the intralamellar space was greatly enlarged. Notice the connective tissue (ct) with isolated microfibrillae in the ground substance. The outer lamella (L)of the envelope is attached to the connective tissue ground substance. In the wide interlamellar space one notices particles which may have separated from the surface of the cell. They may indicate changes in the cell surface which could be responsible for the apparent absence of the inner lamella of the envelope in this case.

The connective tissue ground substance is of a very low electron density although it is dense enough to differ from the area of the interlamellar space. A high degree of hydration of the connective tissue is indicated both by the low density of the ground substance and by the isolation of the microfibrillae, which are no longer bundled into the reticular fibers that surround the fat cells in silver-stained light microscopic sections. This, together with the obvious accumulation of fluid in the interlamellar space, suggests that the impressive changes in the envelope that accompany the release of fat are due to a rapid change in the water content of the fat cell.

It is of special interest that the outer lamella of the envelope remains in contact with the connective tissue ground substance when it recedes from the cell; we may say it forms the surface of the ground substance.

Conclusions

Electron microscope observations of the surface of the fat cell show that the cell wall which appears in light microscope sections of adipose tissue has a well-marked line or seen from above as a distinct membrane, contains not only an outer layer of connective tissue and an inner layer of cytoplasm, both of varying thickness, but also the two lamellae described here together with the interlamellar space, as the envelope of the fat cell. The outer lamella is obviously continuous with the connective tissue ground substance and the inner lamella with the cytoplasm; thus the former is a surface layer of the limiting membrane of the ground substance and the latter is the surface structure of the cell or the true cell membrane. Although these membranes are different in kind we feel justified in considering them, together with the interlamellar space, as a functional unit which as a whole is instrumental in the transport of the material involved in the metabolism of the fat cell.

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PROGRESS REPORT: STUDIES OF REGENERATING MAMMALIAN LIVER

I. Changes in Nucleic Acids Following Partial Hepatectomy in Animals Previously Subjected to Repeated X-irradiation

A. D. Barton and Anna Kane Laird

In previous experiments[1] we found that massive administration of amethopterin following partial hepatectomy did not inhibit the synthesis of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein that takes place during regeneration of the liver. However, when a moderate daily dose of amethopterin was administered for five days preceding partial hepatectomy, the synthesis of all of these compounds was inhibited.

In the present experiment, male Holtzman rats (350-450 g) were subjected daily to whole body X-irradiation, (100 r, 250 r, 400 r), for 5 days preceding partial hepatectomy, and the synthesis of DNA and RNA were studied during the subsequent regeneration of the liver. The animals were killed 28 hr after the operation; 3 hr before sacrifice, each animal received 15 μc of inorganic P^{32} by intraperitoneal injection. The procedures for isolating samples and determining the quantities and relative specific activities of the nucleic acids have been described previously.[1]

The principal results of this experiment are given in Table 9. Considering first the DNA, one would expect partial hepatectomy of rats having 32 mg of DNA per whole liver to leave a liver remnant containing approximately 11 mg of DNA, and this is what was found 28 hr after the operation in the animals of the 400 r group. In the hepatectomized animals that had received no X-irradiation, 14.4 mg of DNA was found, suggesting that some synthesis of DNA had taken place. Intermediate values were found in the other X-irradiated groups. When the nonirradiated animals were subjected to partial hepatectomy, the relative specific activity of the DNA rose from 1.21 to 4.04; the corresponding values after partial hepatectomy of the X-irradiated animals showed much smaller increases. Thus, on the basis of both the quantity and the labeling of the DNA it is evident that the X-irradiation inhibited the synthesis of DNA.

The results for RNA were quite different. By 28 hr, the total RNA per liver in nonirradiated animals had increased from approximately 40 mg estimated to be present immediately after the operation to 57 mg of RNA. The corresponding increases in the X-irradiated animals were distinctly larger. Moreover, the radioactive labeling of the RNA after hepatectomy in the X-irradiated animals was quite comparable with that

TABLE 9

Changes in nucleic acids during liver regeneration following partial hepatectomy in animals previously subjected to repeated X-irradiation

	DNA			RNA			RNA/DNA	Total cells per liver ($\times 10^{-9}$)
	pg/nuc*	mg/liver*	Rel S A **	pg/nuc*	mg/liver*	Rel S A **		
C***	15 1	32 4	1 21	51	110	4 57	2 00	2 17
H	18 9	14 4	4 04	75	57	9 89	2 36	0 76
X100rH	24 0†	14 2	2 64	116†	69	7 40	2 87	0 59†
X250rH	24 9†	12 4	2 38	138†	68	8 97	3 28	0 50†
X400rH	17 0	11 9	1 75	86	60	8 68	2 97	0 70
X400r	16 5	29 8	1 93	52	94	6 37	1 86	1 80

* Quantities of nucleic acids are expressed as picagrams ($g \times 10^{-9}$) per nucleus and as milligrams per liver

** Relative specific activities are expressed as $\frac{\text{counts per second per mg nucleic acid}}{\text{counts per second per } \mu g \text{ acid soluble phosphorus}}$

*** C, control, H, partial hepatectomy, X, X-irradiated 100 r, 250 r, 400 r, daily dose administered for 5 days prior to partial hepatectomy (250 kv, 15 ma, 0 25 mm Cu, 1 mm Al, half value layer 1 1 mm Cu, 125 r per min)

All measurements were made on pooled material from 4 animals except for group X400 r which consisted of 2 animals

† These values are questionable because they are based on nuclear counts believed to be erroneously low, possibly due to failure of some fragile nuclei to survive homogenization.

of the corresponding nonirradiated controls. It is evident that the previous X-irradiation resulted in increased synthesis of RNA following partial hepatectomy, and this is borne out by the values for the ratio of RNA to DNA.

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A NOTE ON THE STATISTICAL DETERMINATION OF SHAPE OF CHROMATIN ELEMENTS IN CROSS SECTIONS OF NUCLEAR MATERIAL IN HUMAN SPERM

Bernard R. Nebel, Sylvanus A. Tyler, and Carol J. Murphy

The interpretation of electron microscope images showing particulate chromatin in human spermatids (obtained at biopsy from an infertile male) presented a problem of three-dimensional abstraction based on the two-dimensional projection of the microphotographs. A method published by Smith and Guttman[1] was adapted to meet this problem. The method relates the physical measurements of a plane section with the surface-to-volume ratio of its three-dimensional counterpart. A grid of parallel lines, separated by distances greater than the diameter of the largest particle, is superimposed on the original photograph, and the total number of intersections of grid lines with particles is counted. Using the number of intersections ($\bar{N}$) (twice the number of double intersections plus the number of single or tangential intersections), the area (A) occupied by the particles evaluated, and the distance between grid lines (d), the surface to volume ratio (S/V) can be calculated from the formula

$$\frac{S}{V} = \frac{2\,\bar{N}d}{A} \qquad (1)$$

This derived value of S/V is then compared with the theoretical S/V values for the sphere, prolate spheroid, and cylinder to determine the probable shape of the examined elements.

In the human sperm the chromatin particles were found to approximate a spherical shape; this is considered to result from the pathological condition involved.

As a test of the method, a photograph of a Tradescantia microspore nucleus in early prophase was similarly evaluated. The chromatin particles in this material were shown by this method to be cylindrical. As is known from past work, they are actually sections of spirals. Thus, the present method bears practical value for other problems of this kind.

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PRELIMINARY REPORT: TOPOLOGICAL BIOLOGY

Ernesto Trucco

Some authors have emphasized the fact that the conventional mathematical approach to biology might prove very useful within a limited range of applicability (e.g. in the construction of models), but would probably fail to bring out those interrelations of functional and structural elements usually referred to as "organization" (Reference 1, pp. 12-15, p. 183). In a wider context we may recall the brief reference to "Principia Mathematica" at the beginning of Woodger's "The Axiomatic Method in Biology,"(2) where we are reminded that "numerical relations derived from measurements constitute the subject matter of a very special development of mathematics," so that many other "purely abstract sciences, with their laws, their regularities, and their complexities of theorems" could possibly be used to represent natural phenomena. Woodger's book itself constitutes a noteworthy attempt in this direction.

Or, to quote from Bertalanffy (Reference 1, pp. 158-159): "Many of the most essential questions of biology are not a matter of quantities but of 'pattern,' 'position,' and 'shape.,'" and; "These are questions that, as far as can be seen, are partly related to topology and analysis situs; that is, they concern problems of relations within manifolds."

About 25 years ago B. Bavink, inspired by the monumental work of the Finnish philosopher Hermann Friedmann, advocated the use of a "Gestalt" - mathematics which would subordinate the notion of quantity to that of structure (or form, or "Gestalt"). His goal, merely outlined in a few sentences, was to obtain a theory of natural phenomena in which purely biological facts would appear as relational properties, whereas physical laws (including those parts of biology which can be accounted for by physics and chemistry) would be included as metric properties, i.e. as limiting or degenerate cases of the laws governing the general form factors. Thus physics would, in some ways, become a very special branch of biology. This possibility was later mentioned independently by Rashevsky (Reference 3, p.345). Bavink's ideas, together with brief but useful summaries of Friedmann's work are given in References 4;5, p. 629; Reference 6, pp. 388-89; and Reference 7, pp. 34-36. For more details the reader should consult Friedmann's own books: Reference 8 and in particular Reference 9, Chapters 10-14.

In the field of psychology, F. H. Allport(10) published a paper in which he stresses the importance of structural relations (as opposed to, or complementary to, metric relations). His paper actually outlines a "theory of structure," and the same theme is taken up with broader views in a recent book by the same author (Reference 11, pp. 19, ff and Chapter 21).

As we have seen, L. von Bertalanffy,[1] and also Bavink,[7] include topology among the mathematical disciplines which deal primarily with structural relations and could thus be of use to the biologist. It is true that Waddington (Reference 12, p. 512), has warned us - and rightly so - against a naive application of the topology of surfaces to problems of morphology. Nevertheless, some results of possible biological significance can be obtained using topological concepts, as evidenced by the work of Rashevsky[3] (See also several other papers which have appeared in the Bulletin of Mathematical Biophysics since 1955 and the third edition of his "Mathematical Biophysics," to be published in the near future). A summary of Rashevsky's main results is given by D. Jordano Barea (Reference 14, pp. 6-7). Jordano himself[13,14] has made interesting contributions independently in this field. We should also mention a recent paper by R. Rosen[15] which may be classified as belonging to "Systems Theory."

In contrast to some of the authors quoted above, who limited themselves to statements of a general nature, Rashevsky and Jordano made a concrete attempt to apply topological ideas to the formulation of biological problems. In his earlier papers N. Rashevsky represented abstract properties of an organism as points of a plane and connected them by appropriate arrows (thus obtaining a directed graph) to show their interrelationships. He then considered various possible mappings, or transformations, between such graphs pertaining to different organisms.

More recently the same author made use of set-theoretical topological methods. The idea behind this approach is that sets of biological properties (considered as collections of abstract objects) may be investigated by means of the mathematical theory of sets. In particular, if suitable neighborhoods can be introduced, the sets acquire the attributes of topological spaces. This last assumption may, however, be too restrictive (Rashevsky, private communication). Whereas the properties in question have the character of biological functions in Rashevsky's papers, Jordano is guided in his choice rather by considerations of morphological or functional homologies (in the biological sense) between organisms. Unfortunately, the two papers which Jordano has kindly sent us so far are of expository nature and give only a few details. For example, it is not quite clear in what sense statements such as "Every living being or set B is a lattice," and "every pair of elements $a \subset B$, $b \subset B$, has a greatest lower bound" should be understood.

However interesting the general direction of this research may be, it is still of a very tentative nature Mathematicians have obtained an imposing array of important theorems, but it is an open question whether these can be applied directly to the problems in which we are interested.

In our opinion one of the first tasks will be a careful enumeration and analysis of the properties to be considered since these, after all, will play the role of points in our sets. In this connection we should quote an interesting paper recently published by C. F. Ehret.(16)

We have in mind a synthesis of Rashevsky's and Jordano's approaches. As we indicated above the first of these is, roughly speaking, more physiological, and the second more morphological. A further line of inquiry which we wish to pursue is Jordano's conception of the organism as a topological complex. Merely as a possibility we suggest the following procedure. consider, for a given organism, certain subsets M_1, M_2, . . . of biological properties. The elements of M_i are properties P_{i1}, P_{i2}, ... which can be accurately detected and specified for the organism in question; they may be of quite different nature and, in a more refined description, certain subproperties of P_{ij} should be counted as separate elements of M_i. With each subset M_i associate a point a_i; then the set $(a_\alpha, a_\beta, \ldots)$ will be considered as a simplex if and only if the collection of subsets $(M_\alpha, M_\beta, \ldots)$ has a non-empty intersection. In other words, we construct the abstract complex which a topologist would call the "nerve" of the collection of sets $\{M\}$ (Reference 17, p. 160). In this way, however, we obtain different complexes depending on how we choose the subsets M_i. It is, therefore, necessary to establish some criterion for that choice. We can, for example, assume that each M_i represents a set of properties which belong together as explained by Rashevsky in Reference 18, p. 270.

If this identification of organisms with complexes is feasible we naturally expect that the sets M_i corresponding to primitive organisms contain relatively few basic properties, whereas higher organisms will show greater richness and variety of such properties. The interrelations between biological functions may then be specified by incidence matrices of complexes rather than by directed arrows as in Rashevsky's graphs.

Jordano himself does not explain in detail how to realize the isomorphism between topological complexes on one hand and living organisms on the other hand. In his case the vertices of each complex correspond to the constituent parts of the organism; the choice of these vertices, as biological cells, or tissues, or organs, etc., gives rise to a theoretical classification of organisms (See Table 1, p. 12, Reference 14).

We believe that Jordano's ideas can be successfully applied with the above-mentioned modifications, and that it will be possible in this way to rederive the results which Rashevsky obtained from his use of plane graphs. The principle of biotopological mapping (Reference 3, p. 325) would, of course, apply equally well to transformations between abstract complexes. The approach suggested here, however, has the advantage that it is more closely related to the set-theoretical method and, at the same time, it introduces the notion of simplex in a natural way. We think it important to continue these studies and hope to report a few more concrete results in later communications.

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PROGRESS REPORT: DEUTERIUM OXIDE INTOXICATION IN RATS

II. Enzymatic Dehydrogenation of Tetradeuterosuccinate

John F. Thomson, T. Richard Sato, John H. Pomeroy, and Carolyn A. Craig

Experiments with tissues from rats fed heavy water have not been conclusive in localizing any biochemical lesions which may result from deuteration. Of a number of enzymes in livers and kidneys of deuterated animals which have been assayed, only catalase and esterase have shown a significant decrease.[1] However, little has been done on the metabolism of deuterated substrates. This report deals with the enzymatic dehydrogenation (more precisely "dedeuteration") of completely deuterated succinic acid.

Tetradeuterosuccinic acid was prepared by catalytic deuteration of acetylenedicarboxylic acid. The compound contained >96 atom per cent deuterium;* no evidence of C-H bonds was detectable in the infrared absorption spectrum.

Preliminary results have been obtained on the behavior of tetradeuterosuccinate in the succinoxidase system. Rat kidney homogenate and an extract of beef heart[2] were used as sources of the enzyme. It is evident from Table 10 that the rate of dehydrogenation of tetradeuterosuccinate

TABLE 10

Enzymatic dehydrogenation of succinate and tetradeuterosuccinate

Enzyme Source	Acceptor	Per cent control activity*	
		Succinate-D_4 in H_2O system	Succinate-D_4 in D_2O system
Rat kidney homogenate	Cytochrome c	47	20
Beef heart (Slater)	Methylene blue	54	-
Beef heart (Slater)	Phenazine methosulfate	-	16**

*Control activity is the rate of dehydrogenation of hydrogen-containing succinate in an H_2O system.

**Beef heart preparation[2] washed and resuspended in D_2O; no inhibition observed when tested with hydrogen succinate in H_2O system.

*We are indebted to Dr. Henry Crespi of the Chemistry Division for this determination.

was only half as rapid as that of ordinary succinate when tested in the usual assay systems. In a test system made up with D_2O instead of H_2O, in which the only hydrogen atoms present were those in non-exchangeable positions of the enzyme and the acceptor, the rate of dehydrogenation was only one-fifth of the rate observed in the hydrogen system.

Attempts have been made to prepare deuterofumarate enzymatically from tetradeuterosuccinate. Since it is necessary to carry out the reaction in a completely deuterated system to prevent enzyme-catalyzed exchange,[3] the rate of the reaction is very slow unless large amounts of enzyme are used. Yields of deuterofumarate have been disappointingly small.

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RADIATION-INDUCED GENETIC EFFECTS IN SUNFLOWER AS INFLUENCED BY BORON NUTRITION

John Skok

It has been shown[1] that in the sunflower the state of boron nutrition of the plant has a marked effect on radiosensitivity. The development of radiation injury was markedly alleviated by withholding boron for short intervals prior to X-irradiation with doses that normally produce pronounced radiation symptoms. Apparently the temporary absence of boron resulted in retardation of cellular activity and arrested growth, and thus rendered the plants more radioresistant.

After these effects had been noted, experiments were carried out to determine whether withholding boron for short periods prior to X-irradiation might also reduce radiation-induced genetic effects in the succeeding generation. One-week-old sunflower seedlings were grown in nutrient solutions with or without boron for 3 days, X-irradiated with 1000 r, and then immediately transferred to a boron-containing substrate, following the procedures previously described.[1] The plants were maintained in a controlled-environment growth room for about 2 weeks and were then transplanted to the field along with unirradiated plants. Seeds were collected from these plants and were sown out in a series of plantings. Over 2000 individual F_1 plants were observed and scored for abnormalities.

All the F_1 plants from unirradiated parents as well as all the F_1 plants from parents of the irradiated lot in which the plants were deprived of boron for three days prior to irradiation were normal in respect to observable leaf characteristics. Leaf abnormalities did appear in F_1 offspring from irradiated parents that were not deprived of boron prior to radiation. Three distinct types plus a fourth one composed of a combination of two of the types were expressed: (1) marginal serration and leaf distortion, (2) a narrow-leaf condition, (3) a combination of the narrow-leaf condition and marginal serration, and (4) a downward rolling of tips and margins.

Of the total number of observed F_1 plants derived from parents irradiated in the seedling stage and not deprived of boron, 5.6% exhibited leaf abnormalities. Among the lots (of this same treatment group) that contained abnormal F_1 plants, a mean of 11.2% of the F_1 individuals expressed abnormalities. A test of this observed effect of withholding or supplying boron prior to X-irradiation on the expression of abnormal leaf characters in the following generation is highly significant ($P = << 0.001$).

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CHEMICAL PROTECTION. THE COMPARATIVE EFFECTIVENESS AGAINST FISSION NEUTRONS AND Co^{60} GAMMA RAYS OF AET ADMINISTERED ORALLY TO MICE BEFORE IRRADIATION

Howard H. Vogel, Jr., Norman A. Frigerio, and Donn L. Jordan

Pretreatment with AET (S, 2-aminoethylisothiuronium bromide hydrobromide) has been shown to protect mammals against the effects of X-radiation.(1) For maximum benefit, the compound must be given less than one hour before irradiation; it has no protective or restorative effect if administered after irradiation. Most of the experiments reported to date have been carried out on mice or rats irradiated with X-rays or γ-rays. Early "intestinal death" following acute exposures is characteristic of the response to fission neutrons but not of the response to X-rays or γ-rays, and this has suggested differences in the mechanisms of injury.(2) The present experiments were initiated to compare the effectiveness of this chemical agent against fission neutrons and Co^{60} γ-rays.

The animals used were young adult female CF No. 1 mice. AET was administered orally in a dose of approximately 600 mg/kg of body weight; each mouse received 0.34 to 0.5 cc at pH 7.4 in acetate buffer. Preliminary toxicity trials indicated that this was not a lethal dose although it resulted occasionally in diarrhea.

In the first experiment, 64 mice were divided into two equal groups; one received AET 10 min before irradiation and the other served as the control. All 64 mice were given a single, whole-body exposure to 350 rads of fission neutrons, which is usually a $LD_{90/30}$ under our experimental conditions. Only 53% of the AET-treated mice died within 30 days, in contrast to 85% of the controls (Figure 18). The $LD_{50/30}$ following a single exposure to fission neutrons at CP-5 is 323 rads; hence AET in effect reduced the neutron dose from 350 rads to about 325 rads, or reduced it by about 7%. This is certainly far less than the "protective factor of about 2" reported for mice exposed to X-rays.(3)

Experiment 2 consisted of two replicate exposures to Co^{60} γ-rays, carried out at different times. After exposure to 1060 rads, all of the untreated mice, but only about 25% of the AET-treated mice, died within the acute period (Figure 19). The 25% value is that to be expected from a single dose of 815 rads, hence the dose reduction was 245 rads, or about 23%.

It is interesting to compare these results with those of similar experiments using cysteine.(4) Pretreatment with cysteine conveyed significant protection against the lethal effects of both γ-rays and neutrons,

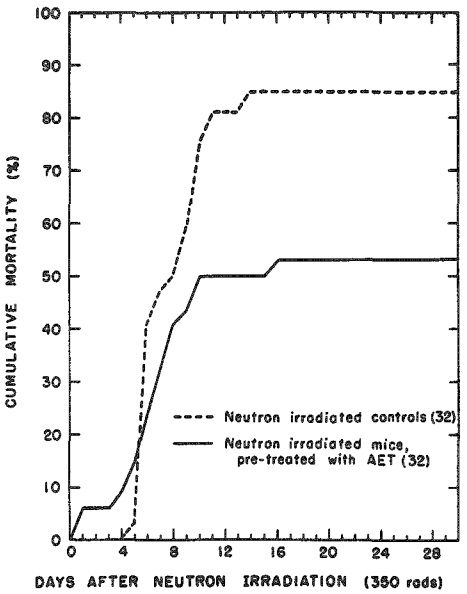

Figure 18. Cumulative mortality in CF No. 1 female mice given 350 rads of fission neutrons, with or without pretreatment with AET.

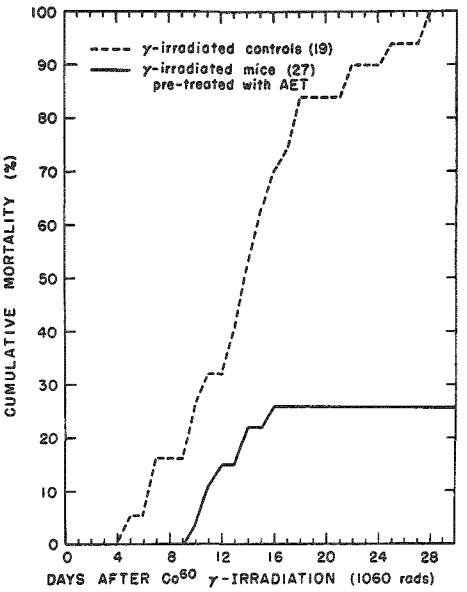

Figure 19. Cumulative mortality in CF No. 1 female mice given 1060 rads of Co^{60} γ-rays, with or without pretreatment with AET.

but the dose reduction observed for neutrons (7.2%) was only about half that observed for γ-rays (14.9%). It would appear from these experiments that cysteine and AET were approximately equivalent in protective effect when given to mice prior to neutron irradiation, but AET was slightly more effective against γ-rays.

AET apparently does not significantly protect mice from the early intestinal syndrome, 4-10 days after fission neutrons (Figure 18). Doherty and Burnett[1] reported 20 to 30% survival in AET-protected mice irradiated with 600 rads of 14-Mev neutrons (3-hr exposure).

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RADIATION RECOVERY: FRACTIONATION OF Co^{60} GAMMA-IRRADIATION WITH BRIEF INTERVALS (MINUTES) BETWEEN PAIRED EQUAL DOSES TO 4-DAY CHICK EMBRYOS IN OVO

Howard H. Vogel, Jr., and Donn L. Jordan

We have shown previously[1] that for early (6-hour) death in the 4-day chick embryo, the LD_{50} of Co^{60} γ-rays is approximately 1191 ± 31 rads. The lethal range was found to be 1050-1500 rads. The early death was associated with hemorrhages in both the intra- and extra-embryonic blood vessels.[2]

In the present series, 4-day chick embryos were first exposed to single doses of 1500 rads of γ-rays. This was expected to be an LD_{85} for the early mortality, but in these exposures was actually an LD_{70}. Then, using the exposure techniques previously described,[1] embryos were exposed in ovo to two doses of 750 rads each, separated by an interval of a few minutes during which the eggs were kept warm. The dose rate in all of these exposures was 10 to 11 rads/min; thus, the single irradiation required 145 min, and each half of the split exposure required 72.5 min.

The results are illustrated in Figure 20. When the interval between the exposures exceeded 20 minutes, the 6-hour mortality did not occur. In fact, there was little evidence of the petechiae and hemorrhages usually observed after single doses of 1500 rads. It is clear that even as brief an interval as 8 to 15 minutes between the two exposure periods of 72.5 minutes was sufficient to allow a majority of the embryos to survive the early mortality.

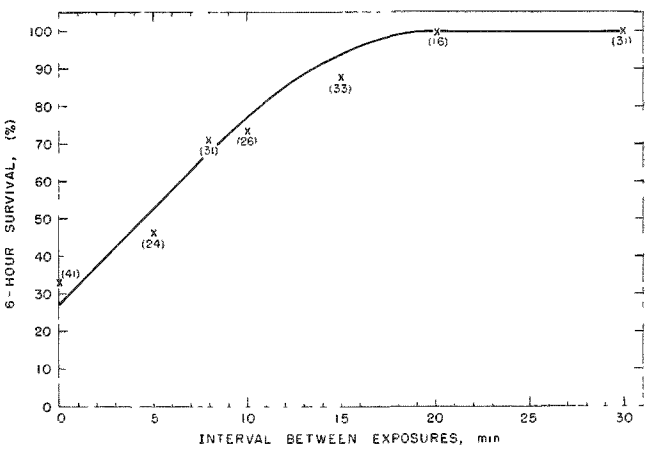

Figure 20

Recovery from early (6-hour) radiation death in 4-day chick embryos given equal paired doses (750 rads) of Co^{60} γ-rays separated by brief intervals (minutes). Figures in parentheses give the number of embryos tested at each point.

This study will be continued to examine further the mechanisms of injury and repair in the chick embryo.

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PILOT STUDY OF SUBACUTE IRRADIATION FOR FUTURE FAST NEUTRON AND GAMMA-RAY EXPOSURES

II. Longevity after 13 Daily Exposures

Howard H. Vogel, Jr., and Donn L. Jordan

The present pilot study was undertaken to evaluate the effects of a series of fractionated doses from the 30-day acute dose range of fission neutrons and γ-rays through the subacute area to low total doses. The information should be useful in planning a larger experiment in the chronic range. The schedule of exposures to both radiations has already been described.(1)

The animals used were CF No. 1 female mice, 10 weeks old. The animals in Group 6 of each irradiation category showed the mortality pattern associated with acute single exposures. The survivors of all groups were observed to determine longevity and incidence of cataracts, lymphomas, and other tumors. Mean survival times for all groups are listed in Table 11. (See also Figure 21).

TABLE 11

Mean survival times of mice given 13 daily exposures to small doses of fission neutrons or Co^{60} γ-rays

Neutron-irradiated				Gamma-irradiated			
Group	No. mice	Daily dose, rads	Mean survival time, days after irradiation	Group	No. mice	Daily dose, rads	Mean survival time, days after irradiation
0	36	0	568	0	32	0	595
1	36	2.2	563	1	24	6.3	516
2	18	4.4	459	2	18	13	417
3	36	9.0	382	3	24	25	406
4	18	17.5	377	4	18	52.5	223
5	36	36	189	5	24	108	50
6	18	70	16.0	6	18	210	14.3

At the present time there is little evidence from these data to indicate any marked increase in relative biological effectiveness (RBE) (n/γ) above the figure following acute exposure (Table 12). There have been indications from other work that the relative biological effectiveness (n/γ) is elevated significantly in animals exposed to chronic levels of radiation for the length of their lives. However, the present conclusion is that 13 brief daily doses of these radiations do not produce the same effects as long-continued chronic exposures.

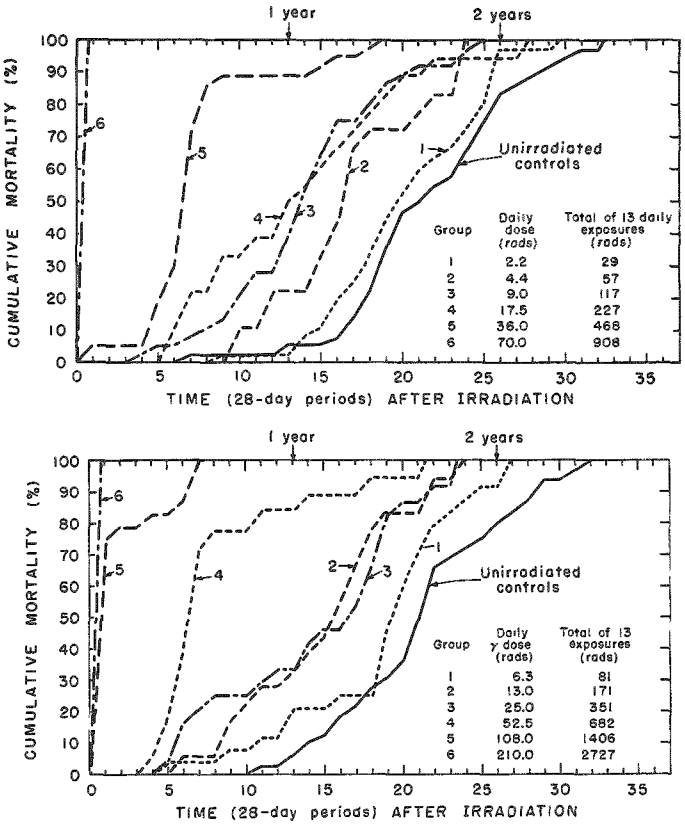


Figure 21

Cumulative mortality in CF No. 1 female mice after 13 daily exposures to small doses of fission neutrons (upper graph) or Co^{60} γ-rays (lower graph). The mean survival times for these groups are given in Table 11.

TABLE 12

Estimated RBE (n/γ) based on survival in the experimental groups

Daily neutron dose, rads		Daily γ-ray dose, rads	Estimated RBE
2.2	is less effective than	6.3	? - 2.9
4.4	is less effective than	13	
	but more effective than	6.3	1.5 - 3.0
9	is less effective than	52.5	
	but more effective than	25	2.6 - 5.8
17.5	is less effective than	52.5	
	but more effective than	25	1.4 - 3
36	is less effective than	108	
	but more effective than	52.5	1.4 - 3
70	is less effective than	210	
	but more effective than	108	1.4 - 3
323	Single acute dose, $LD_{50/30}$	902	2.8

The effects of 13 daily exposures to Co^{60} γ-rays and to fission neutrons on life span of CF No. 1 female mice are shown in Figures 22 and 23. Although the two curves are not identical in shape, there seems to be an indication in both of an exponential relationship between the total dose and the per cent life shortening.

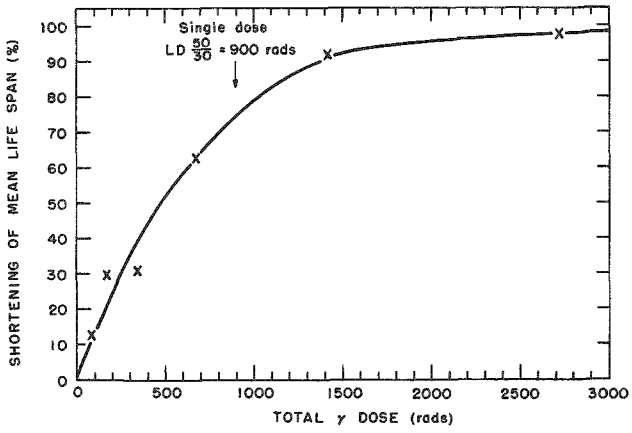
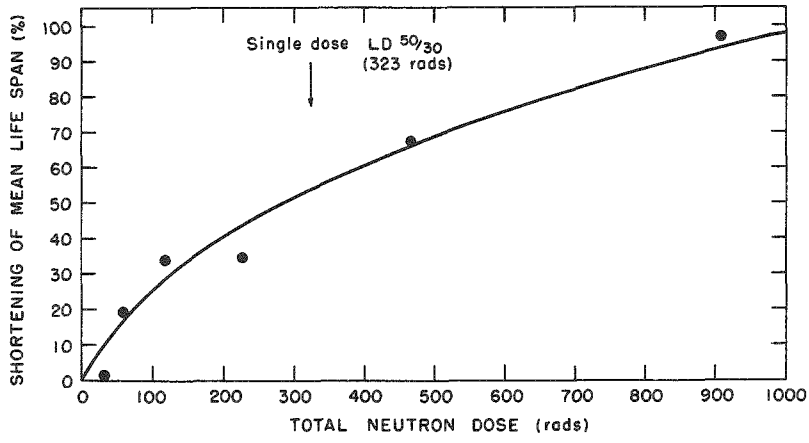


Figure 22

Effect of total accumulated dose of Co^{60} γ-rays (13 daily exposures) on life span in CF No. 1 female mice.

Figure 23

Effect of total accumulated dose of fission neutrons (13 daily exposures) on life span in CF No. 1 female mice.



In Table 13 are presented the comparative doses of the two radiations found necessary in this experiment to shorten the life span of the CF No. 1 female mouse by specified percentages. Fission neutrons appear to be from 1.5 to 2 times as effective as Co^{60} γ-rays in life shortening. Thus the relative biological effectiveness (RBE) for these two radiations appears to be lower for late effects of fractionated exposures than for acute single exposures.

It is of some interest that Storer[2] has reported that a single dose of 600 rads of either X-rays or thermal column radiation reduced the life span of "White Swiss" female mice by less than 30%. In the data presented here, 600 rads (total dose) of Co^{60} γ-rays reduced the mean after-survival time by 60% and a similar total dose of fission neutrons reduced it by at least 75%.

TABLE 13

Relative biological effectiveness of fission neutrons and Co^{60} γ-rays in reducing the life span of CF No. 1 female mice given 13 daily doses of radiation

Shortening of mean life span, %	Total dose fission neutrons, rads	Total dose Co^{60} γ-rays, rads	RBE
90	840	1360	1.6
75	575	900	1.5
50	285	480	1.7
25	100	200	2.0
10	30	60	2.0

An interesting comparison can be made between the mean aftersurvival times in these experiments (CF No. 1 female mice exposed to 13 exposures to Co^{60} γ-rays at a dose rate of approximately 13 rads/min) and Sacher's data[3] on LAF_1 female mice exposed to Co^{60} at a much lower intensity (6 r and 12 r/day) for the length of their lives. Comparative figures are given in Table 14. It would seem from these data that the injurious process that reduces life in female mice is as effectively stimulated by 13 brief daily exposures as by length-of-life irradiation with Co^{60} γ-rays.

TABLE 14

Aftersurvival of female mice exposed to Co^{60} γ-rays. Data for the LAF_1 mice, which were given duration-of-life exposures, are from Sacher.[3] The CF No. 1 mice were those of the present experiment.

LAF_1*		CF No. 1*	
Dose rate, rads/day	Mean aftersurvival, days	Dose rate, rads/day	Mean aftersurvival, days
0	634 ± 13	0	595
6	525 ± 14	6.3	516
12	417 ± 10	13	417

*The LAF_1 mice were 100 days old at the beginning of irradiation; the CF No. 1 mice were 60-80 days old.

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FERTILITY OF YOUNG ADULT FEMALE CF NO 1 MICE EXPOSED TO 13 DAILY DOSES OF FISSION NEUTRONS (3 - 52 RADS DAILY)

Howard H. Vogel, Jr., Donn L. Jordan, Richard R. Barhorst, and Nicholas Bink

The effects of a single irradiation with fission neutrons on the fertility of the female mouse were described in a previous report.[1] During the past two years a series of experiments has been carried out in which mice were exposed to low daily doses of either fission neutrons or Co^{60} γ-rays in order to compare radiation effects at the subacute and chronic levels with previous work in the acute range. In one such experiment, mice were irradiated daily at the CP-5 reactor with fission neutrons delivered at a low dose rate (less than 1 rad/min) for short periods of time (one hour or less) each day for 13 consecutive days. The low intensity necessary for this experiment was achieved by shutting the boral filter plate which is usually operated in the thermal column in the "100% open" position.[2] Thus, although the reactor was operating at full power (2000 kw), relatively few thermal neutrons reached the uranium converter plate and, consequently, the flux of fission neutrons reaching the animal exposure position was decreased to about one-eighth its usual value.

The schedule of daily neutron doses delivered to each group of mice is listed in Table 15. The groups are arranged in order of total neutron dose received, the first experimental group having been irradiated with the highest neutron dose.

Following the 13th exposure, the mice were placed in breeding cages. Two unirradiated control females and two irradiated females were placed with an unirradiated male mouse. The 5 mice were left together for at least 4 days; then the male and females were separated. Each pregnant female was isolated in a cage and checked several times daily for birth of a litter. Careful count was kept of all the young born. No cage was included in the data presented unless the male in the cage had demonstrated fertility by impregnating one of the females.

Five successive mating tests were carried out during the 8 months after the first irradiation. The data for these tests are illustrated in Figure 24 and reported in some detail in Tables 16 and 17.

Several conclusions are apparent from these experiments.

1. Female mice exposed to daily doses of neutrons as low as 6.5 rads delivered on 9 consecutive days, and then mated with unirradiated males, show a definite decrease in pregnancies (per cent parous) and the number of young born (average litter size).

TABLE 15

Irradiation schedule

CF No. 1 female mice irradiated with daily doses of fission neutrons at a dose rate of 0.9 rad/min

Experimental group	Daily exposure time, mins	Daily neutron dose, rads	Number of consecutive daily exposures	Total neutron dose, rads
A	60	52	13	676
B	30	26	13	338
C	15	13	13	169
			9	117
D	7.5	6.5	13	85
			9	59
E	3.75	3.25	13	42
			9	29

TABLE 16

Fertility tests of neutron-irradiated CF No. 1 female mice

Mating test		1	2	3	4	5
Experimental group		Time after exposure				
		Directly after	2 mo	4½ mo	6 mo	8 mo
A	Number of litters born/ female mice tested	0/4	0/3	0/1	-	-
	Avg. litter size					
B	Number of litters born/ female mice tested	4/6	0/6	0/3	-	-
	Avg. litter size	5.5				
C	Number of litters born/ female mice tested	7/8	0/12	1/6	0/10	-
	Avg. litter size	5.7		4.0		
D	Number of litters born/ female mice tested	6/7	10/14	5/12	1/3	1/5
	Avg. litter size	8.7	5.6	8.4	9.0	3.0*
E	Number of litters born/ female mice tested	7/8	9/13	4/7	1/9	2/9
	Avg. litter size	10.0	9.0	7.2	7.0	7.0

*Died in 5 days

TABLE 17

Fertility tests of unirradiated controls, CF No. 1 female mice

Mating test	1	2	3	4	5
Number of litters born/ female mice tested	20/22	27/44	20/30	12/18	8/14
Per cent parous	91	61	67	67	57
Avg. litter size	9.1	9.4	9.3	9.2	7.3

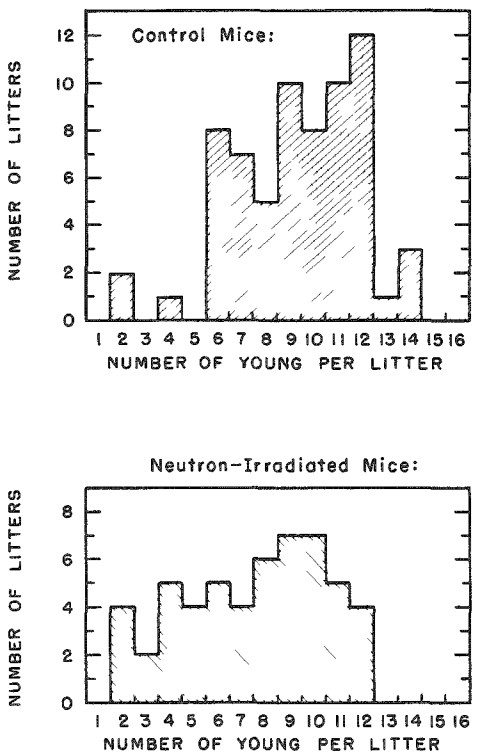

Figure 24

Comparison between neutron-irradiated and control, unirradiated female CF No. 1 mice. Only the data from the first three fertility tests are included (through 4-1/2 months).

Totals:

	Number of mice with litters	% parous	average litter size
Experimentals	53/110	48	7.4
Controls	67/96	70	9.3

2. No mouse receiving thirteen daily exposures of 52 rads (or higher) produced young within the testing period.

3. Mice irradiated with 26 rads daily or less were able to become pregnant and to bear live young, although at the higher exposures, litter sizes were reduced.

4. As the time after irradiation was increased, fewer mice receiving the higher radiation doses were able to give birth to young.

5. At five months after neutron exposure, of all mice receiving more than 85 rads total dose only one litter (4 young) was born.

6. Even at the lowest dose (a total of 42 rads), there was a tendency, after 5 months, toward a decrease in the percentage parous females; there was also a significant reduction in average litter size.

7. From a comparison of the distribution of litter size between controls and neutron-irradiated mice (see Figure 24) it is evident that the irradiated females produce fewer young per litter. Although the range of litter sizes is not very different between the two groups, the number of litters with small numbers is significantly elevated in the irradiated group. Thus 66 per cent of the litters born to control mice contained 9 or more young, as compared to only 43 per cent of the litters of the total experimental group and only 26 per cent of the experimental mice if the two lowest groups (7 and 8) are eliminated from these data.

8. When all irradiated mice that gave birth to young in the first three tests are compared with control females, pregnancy was reduced from 70 to 48 per cent and average litter size from 9.3 to 7.4.

9. In a previous series[1] the average number of young per litter in 124 unirradiated female CF No. 1 mice was 9.7 while the average in this experiment (67 litters) was 9.3.

These mating tests will be continued to determine whether the irradiated females regain their fertility with longer periods after exposure. The data at present do not indicate that this is very probable.

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THE LOCALIZATION OF BORONIC ACIDS IN MOUSE NEOPLASM

Norman A Frigerio and Nicholas Bink

A prime requisite for successful neutron capture therapy[1-4] is the localization of the activatable nuclide within the lesion. Inorganic boron anions have been most used to date although their localization has not been completely satisfactory.[2] The present investigation was undertaken to evaluate organic boron compounds which had been suggested as being possibly more useful The compounds made available to us through the kindness of Drs P G Kruger and H R. Snyder, were the diboronic acid analog of Evans Blue[5] and the monoboronic acid analog of tyrosine [6] In addition borax was included as a reference compound.

Swiss mice were inoculated intracranially with a brain tumor homogenate,[4] and after 10 to 14 days those with obvious brain tumors were selected and injected intravenuously with 0 5 ml of solutions of the appropriate compounds in Ringer solution at pH 7.4. The solutions of the colorless borax and borotyrosine included normal Evans Blue to assist in location of the tumors. Preliminary studies showed that optimum localization with each of these compounds was obtained within 10 to 20 min. Accordingly, 15 min after injection the mice were sacrificed, and the brain and tumor were removed, separated weighed and immersed in a saturated solution of $Ca(OH)_2$ in porcelain crucibles In order to exclude trace amounts of boron this solution was formed in situ by dissolving quartz-distilled calcium metal in quartz-distilled deionized water. The tissues were dried slowly at 100°C then ashed under cover at 550°C.

The resultant ash was analyzed for boron by a micromodification of the curcumin-oxalic acid method [7] Control and duplicate tissue samples analyzed by this method and checked by three independent analytical laboratories employing other chemical and physical methods showed that the method was accurate and reproducible to at least ±5%. Results are shown in Table 18

The disappointingly low values for the ratio of concentrations in tumor and brain especially for the boro-Evans Blue, contrasted markedly with the visible concentration of the dye within the tumor as well as with results obtained by others.[4 8] Accordingly another series of 12 mice were injected intravenuously with 0 5 ml of a 1 per cent boro-Evans Blue solution in Ringer solution at pH 7.4 and sacrificed after 15 min. Brain, tumor and urine were analyzed for boron as above and for the Evans Blue dye moiety by homogenization autolysis and spectrophotometry at 440 540 570 and 650 mμ

TABLE 18

Localization of boronic acids in mouse neoplasm

Compound	No. of mice	Boron per fresh weight, μg				Concentration ratio tumor to brain
		Non-neoplastic brain		Tumor		
		Range	Mean	Range	Mean	
Ringer solution only	7	<0.02	<0.02	<0.02	<0.02	-
1% Borax	8	0.9-2.4	1.3 ± 0.2	2.2-4.9	3.1 ± 0.2	2.4
1% Boro-tyrosine + 0.5% Evans Blue	9	0.6-2.8	1.4 ± 0.3	1.3-9.5	4.1 ± 0.7	2.9
1% Boro-Evans Blue	9	1.0-2.4	1.7 ± 0.2	3.8-7.5	5.5 ± 0.3	3.3

Fresh weight of non-neoplastic brains was 234 to 620 mg with a mean of 373 ± 9 mg.

Fresh weight of tumors was 19 to 284 mg with a mean of 134 ± 14 mg.

In pure boro-Evans Blue the mole ratio of boron to dye moiety should be exactly two. Actual analysis as above gave a ratio of 2.02. In the tumor tissue, however, the ratios obtained ranged from 0.08 to 0.21 with a mean of 0.14. For the urine samples the ratios ranged from 12 to 64 with a mean of 21. Dye concentrations in the brain were too low to permit accurate analyses but, as in the tumor, ratios were all much less than one.

These results suggest that some hydrolytic mechanism, probably enzymatic, is present in vivo for removal of the boron from the dye moiety. Pure boro-Evans Blue is not appreciably hydrolyzed by Ringer solution at pH 7.4 in 15 min, although hydrolysis is appreciable after 1 or 2 days. Thus, the discrepancy between our results and those of Kruger might be explained on the basis of the presence of such an enzymatic system in some strains of mice but not in others. Alternatively, concentration of boron may take place in the growing tumor edge irrespective of enzymatic action. This latter concentration would not be revealed by our analysis of the complete tumor, which was necessary because of the sensitivity limits of the chemical method. Attempts to characterize the boronic acid splitting mechanism in vivo are currently in progress.

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TECHNIQUE OF WHOLE-BODY SHIELDING FOR THE NEUTRON-CAPTURE CHEMOTHERAPY OF TUMORS

Frances E. Knock,* Norman A. Frigerio, and Howard H. Vogel, Jr.

Ingenious methods have been devised for machining boron carbide boxes as body shields for mice bearing small brain tumors which are to be irradiated with slow neutrons. However, the machining of these shields is costly and tedious, and could not be readily applied to a large number of rapidly growing, irregularly-shaped tumors.

For investigation of the neutron capture chemotherapy of a variety of tumors with compounds of B^{10} and the fissionable actinides, a new technique of whole-body shielding has been devised. This involves incorporation of boron or lithium compounds into a soft mass of methyl methacrylate monomer and polymer; this can be made to polymerize at room temperature in a few minutes by incorporation of appropriate quantities of peroxide catalyst and amine promoter. After partial polymerization of the matrix, while the whole remains soft enough for easy working, the shape of the given tumor is outlined on the surface of the shield, and this area is then removed. Final polymerization proceeds, yielding a sheet of hard methyl methacrylate matrix containing the boron or lithium shielding compound and having an appropriately-shaped hole to fit snugly about the tumor. In practice, a group of boxes of shielding compound bound in methyl methacrylate is prepared in advance, and only the tops of such boxes are custom-polymerized to individual tumors before irradiation of the animals. Sides and bottoms of the boxes contain multiple small air holes for circulation of humidified air.

The individual boxes are then fitted into a larger outer assembly made of methyl methacrylate polymer. The dividing partitions, the bottom and the sides are coated completely with the same shielding compound mixture except for multiple air holes. The top of the outer assembly is of clear methyl methacrylate polymer painted with a cross hatch of shielding strips. The strips overlay the edges of the underlying boxes to prevent leakage of slow neutrons to the body of the animal around the edges of the smaller boxes. Air holes in the top and bottom of the larger assembly and in the bottoms and sides of the inner small boxes about each animal are staggered for the same purpose. An example of such an assembly is shown in Figure 25.

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*Dr. Knock is Director of the Knock Research Foundation, Chicago, Illinois.

This work was aided by a research grant to FEK from Presbyterian-St. Lukes Hospital and by the Knock Research Foundation.

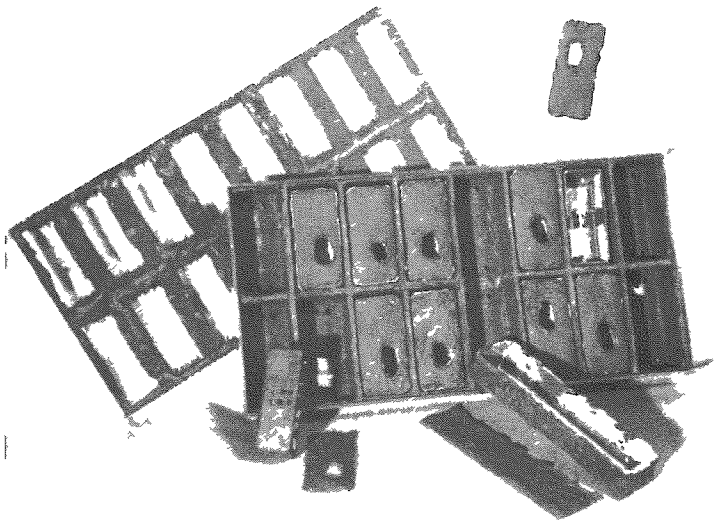

Figure 25. Exposure boxes of methyl methacrylate polymer with boron carbide, in which mammary tumors have been exposed to thermal neutrons, while most of the animal's body is protected from the neutron beam. Note different sized openings which can be fitted to individual tumors.

For our present work with slow neutrons, boron carbide shielding has been found to be most useful. However, the general method could be readily adapted for other forms of irradiation, for example, incorporating compounds for gamma-ray shielding.

The matrix used to bind the shielding compound consists of methyl methacrylate monomer and polymer. Polymerization of the monomer at room temperature is effected by incorporation of tertiary amine promoters and peroxide catalyst. The method is the same as that developed for preparation of fast-setting dental plastics.(1) The polymerization reaction is exothermic and can be accelerated readily by increasing the proportions of promoter and catalyst.

Commercial grade methyl methacrylate monomer, freed of inhibitor by washing with 5 per cent sodium hydroxide, is used. The methyl methacrylate polymer found to be most useful is the 3/60 grade of the L. D. Caulk Company.*

Commercial benzoyl peroxide is used as catalyst and N, N-dimethyl-p-toluidine as promoter. "Norbide" (Norton Company), high-boron, boron carbide, 325 mesh, is used as shielding agent.

*Kindly supplied by the L. D. Caulk Company through John F. Glenn of the Research Department.

For preparation of a typical small batch, 18 g of methyl methacrylate monomer is mixed with 0.7 g of N, N-dimethyl-p-toluidine and 0.7 g of benzoyl peroxide, and the whole is quickly mixed with 9 g of methyl methacrylate polymer. Boron carbide (30 g) is admixed thoroughly, and the mixture is spread out to 2 mm or more in thickness to form sheets of the boron carbide shielding which can be formed as desired. The speed of the reaction can be varied markedly by variation in the proportions of the various ingredients. The use of paper molds has been found to be helpful; the paper can be soaked off after polymerization.

Dosimetry (gold foil measurements) has been carried out on hybrid mice of the Strong AXDBA/212 F_1 strain* bearing transplanted mammary adenocarcinomas 1.0 to 1.5 cm in diameter. The shielding assembly used at present consists of inner and outer boxes constructed of boron carbide-methyl methacrylate mixture, 2 mm thick, as described above. The mice, tranquilized with 7.5 to 15 mg/kg of thorazine IP, are taped in place.

With a flux at the tumor surface of 4.5×10^8 neutrons/cm^2/sec, the flux at the base of the tumors was approximately 20 per cent of the surface flux, while the flux at other portions of the surface of the boron carbide box immediately adjacent to the body of the mice varied from 0.36 to 1.5 per cent of the tumor surface flux.

Reference

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*Kindly donated by Dr. J. Trentin and Mrs. Annabel Liebelt, Baylor University Anatomy Department.

SEQUELAE OF FISSION NEUTRON AND Co^{60} GAMMA IRRADIATION OF MICE

I. Results 325 Days after Exposure

Miriam P. Finkel and Birute O. Biskis

The present experiments were undertaken so that the late effects of internal and external irradiation can be compared in the same test system under similar conditions. Although several excellent fission neutron and gamma exposure studies have been reported, the experimental procedures and animal maintenance routines differed from those employed in our studies of the toxicity of internally administered radioisotopes. Therefore, it seemed advisable to duplicate all the procedures of the isotope experiments with animals exposed to external sources of radiation.

This is a preliminary report through 325 days after exposure. Most of the gross autopsy diagnoses have not yet been verified histologically. Consequently, the tumor data are tentative only.

Materials and Methods

CF No. 1 female mice were shipped from Carworth Farms (New City, New York) when they were 5 to 6 weeks old. Immediately upon arrival they were dipped in Aramite(1) and housed in stainless steel cages,(2) 15 mice per cage. The Aramite bath was repeated a week later. During the first month the animals were observed daily, and a week before irradiation 1779 animals were selected from the stock supply. These were assigned to experimental cages, 15 per cage, by a prearranged schedule based upon a table of random numbers. The mice were then weighed and ear-marked.

During the last week before exposure, animals that showed evidence of ill health were replaced as far as possible from the original stock supply. It became increasingly evident at this time that many had an intestinal infection. The external symptoms were diarrhea, pallor, loss of weight and decrease in normal activity. At autopsy the intestinal tract typically was free of material except for yellowish fluid in the distended caecum and colorless mucus in the very pale, thick-walled colon. The liver was normal. Microscopically, the colon showed a nonspecific inflammatory reaction with more or less ulceration and an increase in goblet cells. These lesions usually were associated with changes in the intestinal flora and fauna, which were examined in dried caecal smears stained with Wright's stain.

In spite of the rather poor condition of the animals, it was decided to proceed with the experiment since the radiation facility would not be available again for at least six months. The disease increased the amount of labor required to keep the animals in relatively clean cages during the first months after exposure, and considerable effort went into testing the efficacy of various antibiotics. Nevertheless, the decision to carry through the experiment proved to be a wise one since only a few mice succumbed to the disease. The symptoms are occurring only infrequently now, a year after irradiation.

The mice were approximately 70 days old when they were exposed to fission neutrons or to Co^{60} gamma rays in the gamma-neutron radiation chamber[3-5] at the CP-5 Research Reactor, which was operated at 2000 kw. The boral filter plate was at the "100% open" position.[6] The exposures were made over a ten-day period. We are greatly indebted to Dr. Howard Vogel and the Neutron Radiobiology Group for their assistance in selecting the dosages and in exposing the animals. We also appreciate the assistance of Dr. Louise Lombard, in the early phases of the experiment.

The dosages and exposure times of the 1755 mice that comprise these experiments are given in Table 19. Level 1 in each instance was selected as being approximately 90% of the $LD_{50/30}$; successive levels were reduced to about one-half of the preceding level. The fission neutron doses, which were read as "n-units" on a standard 25-r Victoreen chamber, were converted to total rads by multiplying by the factor 2.58, which had been determined by Dr. Vogel in collaboration with Dr. H. Rossi of Columbia University for calibrating Victoreen chambers and tissue equivalent chambers at CP-5. The neutron doses listed in Table 19 include 10 to 15% gamma-ray contamination. The Co^{60} gamma-ray doses were measured in roentgens and were converted to rads by the factor 0.97, which was calculated by J. S. Laughlin (Memorial Center for Cancer and Allied Diseases, New York City) for the absorbed dose of Co^{60} gamma rays in a "whole mouse" composed of 69.8% O, 9.8% H, 13.9% C, 4.4% N, 0.75% Ca, 1.3% P, 0.3% K, and 0.2% S.

The routine experimental procedures include daily observations of all mice, monthly weighing and palpation, and the sacrifice with intravenous sodium pentobarbital of moribund animals that, in our opinion, would die naturally within the next 24 hrs. Peripheral blood samples are taken before killing, and roentgenographic examination of the skeleton follows autopsy. Many tissues are regularly studied microscopically, and any additional neoplastic or otherwise interesting areas are included.

TABLE 19

Summary of exposure and tentative results at 325 days

Level	Exposure		Number of mice	Mortality, %	Percentage with tumors of						Tumors per 1000 mice
	Dose, rads	Time, min			Reticular tissues	Mammary glands	Ovaries	Lungs	Other sites	Any site	
Fission neutrons: 7.4-8.3 rads/minute											
1	296.0	40.0	60	66.7	11.7	0	3.3	0	0	11.7	150
2	140.0	19.0	72	45.8	23.6	4.2	2.8	5.6	4.2	33.3	403
3	73.0	9.5	90	26.6	12.2	4.4	5.6	1.1	7.8	21.2	311
4	38.0	4.75	105	22.8	9.5	1.9	0	1.0	3.8	14.3	162
5	20.0	2.38	120	13.3	8.3	0.8	0	2.5	0.8	10.8	125
6	10.0	1.2	150	14.0	7.3	1.3	0.7	1.3	2.0	9.3	127
7	4.0	0.5	180	13.3	6.7	1.1	0	1.7	0.6	7.8	100
Co^{60} gamma rays: 12.3 rads/minute											
1	825.0	66.9	60	100.0	3.3	0	0	0	0	3.3	33
2	388.0	31.5	72	41.7	23.6	1.4	4.2	0	7.0	30.6	361
3	194.0	15.76	90	26.7	17.8	2.2	2.2	0	3.3	21.9	256
4	97.0	7.9	105	21.0	9.5	1.9	1.9	1.0	2.9	13.3	171
5	48.5	3.95	120	10.0	5.8	0	0	0.8	0	5.8	67
6	24.0	1.97	150	10.7	5.3	0.7	0	1.3	0.7	6.7	80
7	9.8	0.8	156	10.9	4.5	0	0	1.3	0.6	6.4	64
Mock exposure: controls											
	0	0.5-60.0	225	8.9	3.1	0.9	0.9	1.3	0.4	4.9	67

Results

Survival. The intestinal infection described above, which appeared before irradiation began, was not highly lethal. The 8.9% mortality among the control animals (Table 19) compares very favorably with other groups of untreated CF No. 1 female mice in our laboratory. In four similar control populations, which included from 120 to 210 animals each, the 325-day mortality values were 3.3%, 19.2%, 29.2%, and 31.9%.

The 325-day mortality values were increased by 38 rads or more of fission neutrons and by 97 rads or more of Co^{60} gamma rays (Table 19). It is interesting that, except for Level 1, the mortality percentages at comparable levels in the two series are quite similar. The mortality rates given in Table 20 vary considerably, and they indicate the extent to which the end point of a cumulative incidence curve can mask the true situation. The variability probably was a consequence of the endemic intestinal infection. After another year has elapsed the mortality rates should be a more useful index of radiation damage.

Tumors. The tumor data in Tables 19 and 20 are based upon gross autopsy observation only. Lesions that may or may not prove to be neoplastic have been included with the unquestionably positive cases, and some tumors of presently undetermined origin have been temporarily grouped according to their most likely site of origin.

Tumors of the blood-forming tissues, mammary glands, and ovaries occurred most frequently among Level 2 and 3 in each series of animals (Table 19). Lung tumors were markedly increased only among Level 2 neutron-irradiated mice. The "other sites" listed in Table 19 include sarcomas and carcinomas of the skin and neoplasms involving the orbit, kidney, liver, and pituitary. These appeared frequently in both series among mice of Levels 2, 3, and 4. The increased incidence of tumors by 325 days after receiving 38 rads or more of fission neutrons or 97 rads or more of Co^{60} gamma rays is emphasized when all sites are combined ("Any site" in Table 19) and when the tumor is used as the basic statistic rather than the tumor-bearing mouse ("Tumors per 1000 mice" in Table 19). It is possible that neutron Levels 5, 6, and 7 also have resulted in an increased incidence of tumors; these values have not yet been subjected to statistical analysis.

That tumors appear earlier among irradiated mice is indicated in Table 20. The morbidity rates will have greater meaning after more data have been accumulated, and they will be examined in more detail at that time.

TABLE 20

Daily mortality and tentative daily morbidity rates per 100,000 mice

Dose, rads	Mortality				Reticular tissue tumors				All other tumors			
	Days 0-100	Days 101-200	Days 201-300	Days 301-325	Days 0-100	Days 101-200	Days 201-300	Days 301-325	Days 0-100	Days 101-200	Days 201-300	Days 301-325
Fission neutrons												
296	500	167	200	400	33	33	120	200	0	0	80	200
140	56	147	259	372	0	103	172	0	0	44	86	372
73	11	79	146	228	0	34	73	116	0	56	73	284
38	19	39	105	188	10	19	40	140	0	10	51	48
20	8	8	76	184	0	8	51	112	0	0	8	112
10	6	40	77	92	6	20	42	32	0	13	28	32
4	6	40	65	52	0	23	41	24	0	6	12	52
Co^{60} gamma rays												
825	933	750	1000	-	0	250	1000	-	0	0	0	-
388	69	119	203	424	0	90	136	256	0	0	85	256
194	22	91	100	332	0	91	75	112	0	0	38	332
97	38	50	115	96	10	50	31	48	0	10	73	0
48.5	25	34	27	36	8	34	9	36	0	0	9	0
24	20	41	28	88	7	20	14	60	0	7	14	28
9.8	6	20	53	84	0	0	33	56	0	0	13	28
Controls												
0	4	4	67	56	0	0	27	20	0	0	18	40

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A STUDY OF THE EARLY STAGES OF COLONY FORMATION FOLLOWING HIGH DOSES OF X-RAYS TO DRY SPORES OF BACILLUS MEGATERIUM

E. L. Powers and Barbara F. Kaleta

An investigation of germination and the early cell division of irradiated and nonirradiated bacterial spores was begun recently to attempt to define more precisely, if possible, the nature of the biological damage that leads to loss of colony formation ability.

Materials and Methods

For this study a further modification of the semi-micro method was necessary.[1] The spores were prepared and mounted on millipore filters (MF's) as described in the previous report. After the desired time of incubation at 35°C, the MF's were removed from the nutrient-containing pads and allowed to air-dry, thereby stopping further development. The discs were put into 0.5% aqueous safranine for 1 min, rinsed with water, blotted, then air-dried for 1 hr. The stained dry MF was placed on a glass slide, soaked with immersion oil to render the MF partially transparent, and covered with a cover slip. Slides prepared in this way may be kept indefinitely.

The ability of the spores to stain with 0.5% aqueous safranine was used as the criterion of germination. Safranine, a basic dye like methylene blue, was preferred because it gave clearer visibility to the stained spores. The full meaning of the stainability of the spore after brief incubation times is unknown; it is not unlikely that it indicates simple development of permeability to the dye. A close correlation has been shown by other authors[2,3] between the different measures of germination: stainability, oxygen uptake, turbidity changes and loss of heat resistance.

By means of a Leitz phase-contrast microscope the number of stained, unstained spores, divided and undivided cells and number of cells per colony in each oil immersion field could be easily ascertained from these slides.

The distribution of spores on the MF is remarkably uniform as shown in Table 21. By using Student's "t" test criterion we find that for MF #139 the probability is that 60% to 70% of the time the difference between the total count for any field and the mean value is due to random sampling; for MF #140, the probability is 30% to 40%, a highly homogeneous distribution.

The X-ray source was the same as described in the previous report and the MF's were irradiated singly at a target distance of 15 cm.

TABLE 21

Random distribution of B. megaterium spores on Millipore Filters

MF #139 Incubation Time = 1 hr Dose (Kr) = 0		MF #140 Incubation Time = 1 hr Dose (Kr) = 0	
Field #	Total Count	Field #	Total Count
1	18	1	23
2	32	2	23
3	24	3	31
4	33	4	24
5	20	5	16
6	18	6	26
7	21	7	18
8	27	8	24
9	27	9	25
10	22	10	23
11	21	11	22
12	29	12	28
13	18	13	19
14	26	14	30
15	28	15	31
16	23	16	18
17	22	17	17
18	28	18	17
19	27	19	25
20	25	20	18
21	28	21	22
22	26	22	19
23	19	23	19
24	29	24	22
25	25	25	27
26	33	26	23
27	21	27	27
28	20	28	27
29	26	29	25
30	16	30	23
24.40 = $\overline{X}$ 0.470 = t*		23.07 = $\overline{X}$ 0.935 = t*	
degrees of freedom = 29 .60 < P < .70		degrees of freedom = 29 .30 < P < .40	

$$*t = \frac{\hat{\sigma} - \sqrt{\overline{\overline{X}}}}{\sqrt{\overline{\overline{X}}/2N}}$$

Results and Discussion

Phase-contrast microscopic observations of the spores after various times of incubation on MF's with nutrient medium showed that it was possible to distinguish the following stages during germination and early cell division of nonirradiated spores:

Stage I. The resting spore. The ungerminated spore does not stain and appears uniformly dark blue and cylindrical with rounded ends. It is visible only under dark-field. The refractile quality of the spore is not observed because of the immersion in oil.

Stage II. The stained spore After 40 min of incubation at 35°C with nutrient medium, 95% to 100% of the spores stain with 0.5% aqueous safranine. They appear uniformly red with no apparent change in size or shape.

Stage III. The stained, swollen spore. After 1 hr of incubation, the stained spores swell and appear diamond-shaped with dark areas near their centers.

Stage IV. The protruding bacteria. After 100 min of incubation, a variety of shapes and sizes are seen, and some bacteria appear to be protruding from the spore walls

Stage V. The vegetative cell. After 2 hr of incubation, vegetative rods are seen, some having already divided. The rods, about 4μ long, are very easily distinguished from the 1-μ spore.

These stages are consistent with those described by Stuy[4] for wet suspensions of B. cereus, if allowances are made for our use of dried preparations mounted in oil

X-irradiated spores follow the same process of germination as the nonirradiated spores during the first hour of incubation (Figure 26). After this time, the survivors in an irradiated sample continue to germinate and divide at about the same rate as the nonirradiated spores up to the point of visible colony formation.

Many of the irradiated spores after 90 min of incubation at 35°C in nutrient medium exhibit loss of stainability, disintegrate, and appear as broken spore coats. That these "lysed" spores are not remnants of surviving vegetative cells is shown by the fact that when they are counted as single spores in a field also containing vegetative cells the total count compares favorably with the control value determined before the appearance of these forms. Figure 27 shows the relationship between the loss of stain (the appearance of "lysed" types) of spores receiving a total dose of 100, 200, and 300 kiloroentgens and the time of incubation. While it is clear that the number of stained spores decreases with time from the maximum at 1 hr, the data are inconclusive in that the relationship to dose is not progressive.

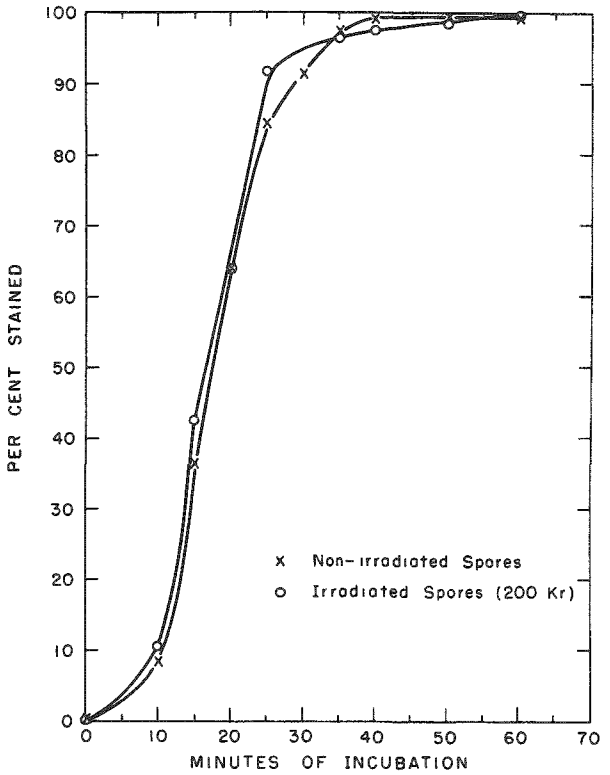

Figure 26. Relationship between time of incubation and percentage of spores that stain with 0.5% aqueous safranine.

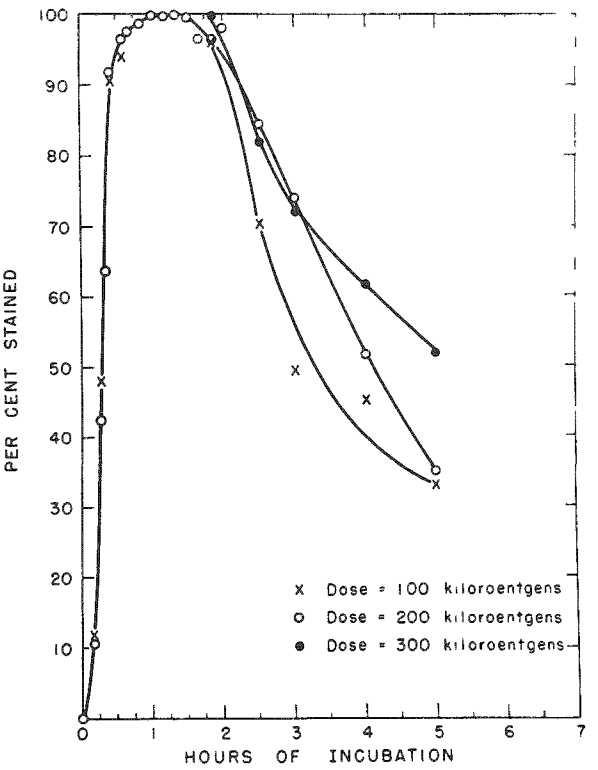

Figure 27. Relationship between time of incubation and percentage of X-irradiated spores that lose their ability to stain with 0.5% aqueous safranine or "lyse."

To ascertain the relationships of dose, number of divisions in microcolonies at different times after beginning of incubation, and number of visible colonies finally produced, MF's containing approximately 5×10^5 spores per MF were irradiated and incubated for either $5\frac{1}{2}$ or $6\frac{1}{2}$ hr, and counts were made of the number of divisions of each colony (Figure 28). The points marked with the number 7 designate the fraction of colonies that have undergone 7 divisions at that time, those marked with the number 6 designate the fraction of those showing 6 divisions plus those that have shown 7, those marked with the number 5 designate the fraction of those showing 5 divisions plus those that have divided 6 and 7 times, and so forth. The line represents a typical dose-response curve for spores irradiated under the same conditions, that is, it was obtained by actual colony counts. These results indicate without doubt that spores may not only germinate but may also divide more than once, and still not form a visible colony. However, the exact number of times a spore may divide and not survive is not indicated. If after $6\frac{1}{2}$ hr of incubation the microcolony has undergone only two divisions, it is not likely that a visible colony will be formed at 7 hr, since about 10 divisions are necessary for visibility. It is not unlikely that 4 to 5 divisions is a number sufficient to determine the ultimate appearance of a visible colony; conversely, an irradiated cell might undergo up to 3 or 4 divisions without giving rise to a visible colony.

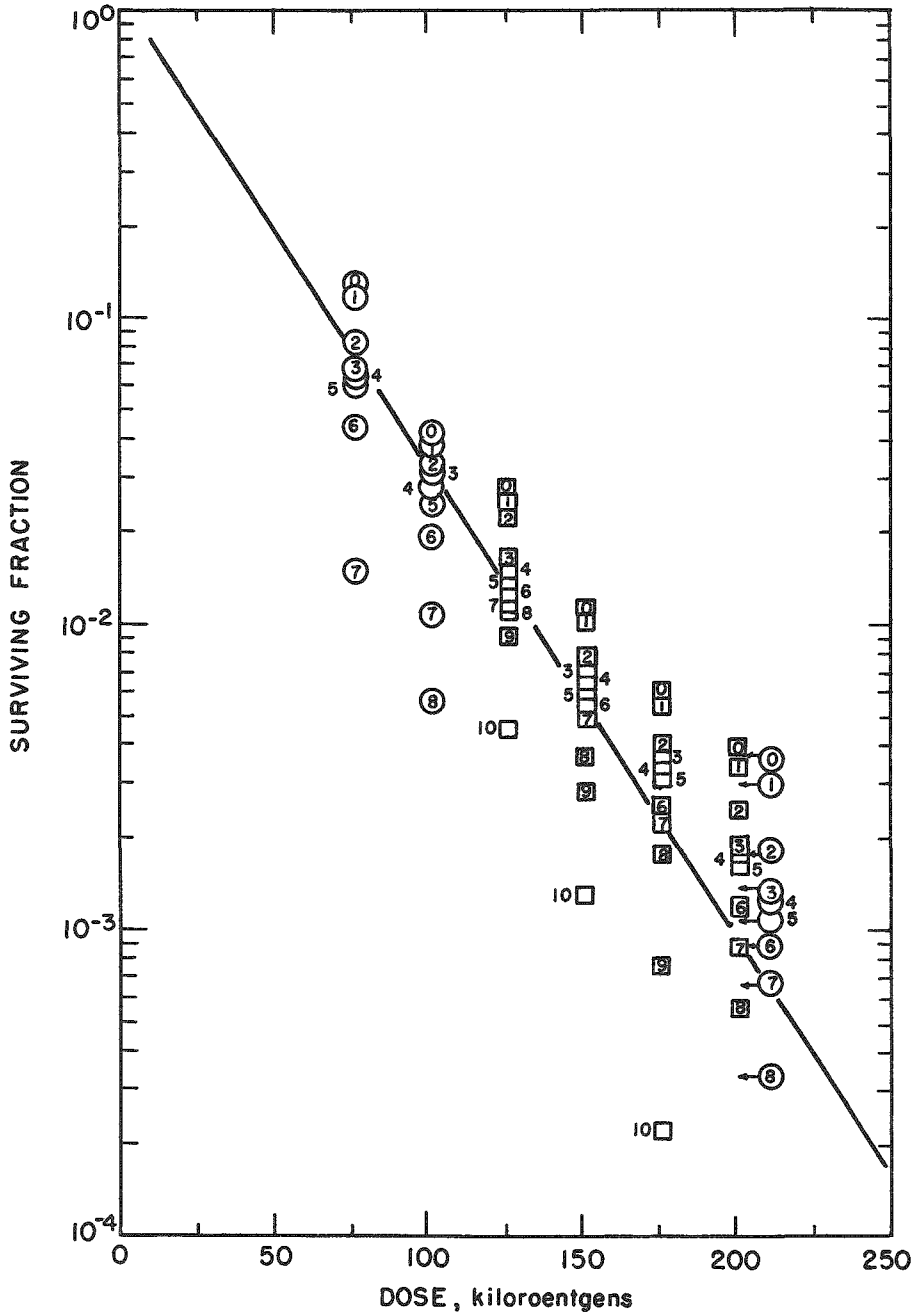

Figure 28. Comparison of a typical dose-response curve with the fraction of germinated spores undergoing the indicated number of divisions. The decreasing number of divisions represent accumulative fractions reading upwards.

Ⓝ 5 1/2 hours Incubation

[N] 6 1/2 hours Incubation

50 kvp, Be window
HVL = 0.070 mm Al
19,860 r/min

Summary

The process of germination has been observed by a micro-method and separated into five stages comparable to those reported by Stuy. It has been shown that spores receiving a dose of X-rays act like nonirradiated spores during the first hour of incubation. This observation is in agreement with observations made by others as reported by Lea.[5] After initial germination, the irradiated "killed" spores may "lyse," stain and swell, form vegetative cells or divide more than once (perhaps three or four times) and still not form visible colonies.

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THE RETENTION OF STRONTIUM-85 IN THE DOG

C. F. Decker and W. P. Norris

The effect of age of an experimental animal upon the retention of certain bone-seeking isotopes has been noted in previous reports.(1) The earlier work with Sr^{85} in rats has been extended to the beagle dog, and this report will be concerned with a description of the experimental plan and results to date.

Fourteen beagle dogs varying in age from 2 weeks to approximately 5 years were injected intravenously with a single dose of approximately 100 μc of Sr^{85}. Immediately after injection each animal was suspended in a plastic sling (Figure 29), and the total-body activity was determined using a NaI scintillation detector and a single-channel pulse-height analyzer. A standard serum vial of solution made from the original injection solution and containing approximately the same activity as that injected into the dog, was then placed in fixed position in the sling and counted.

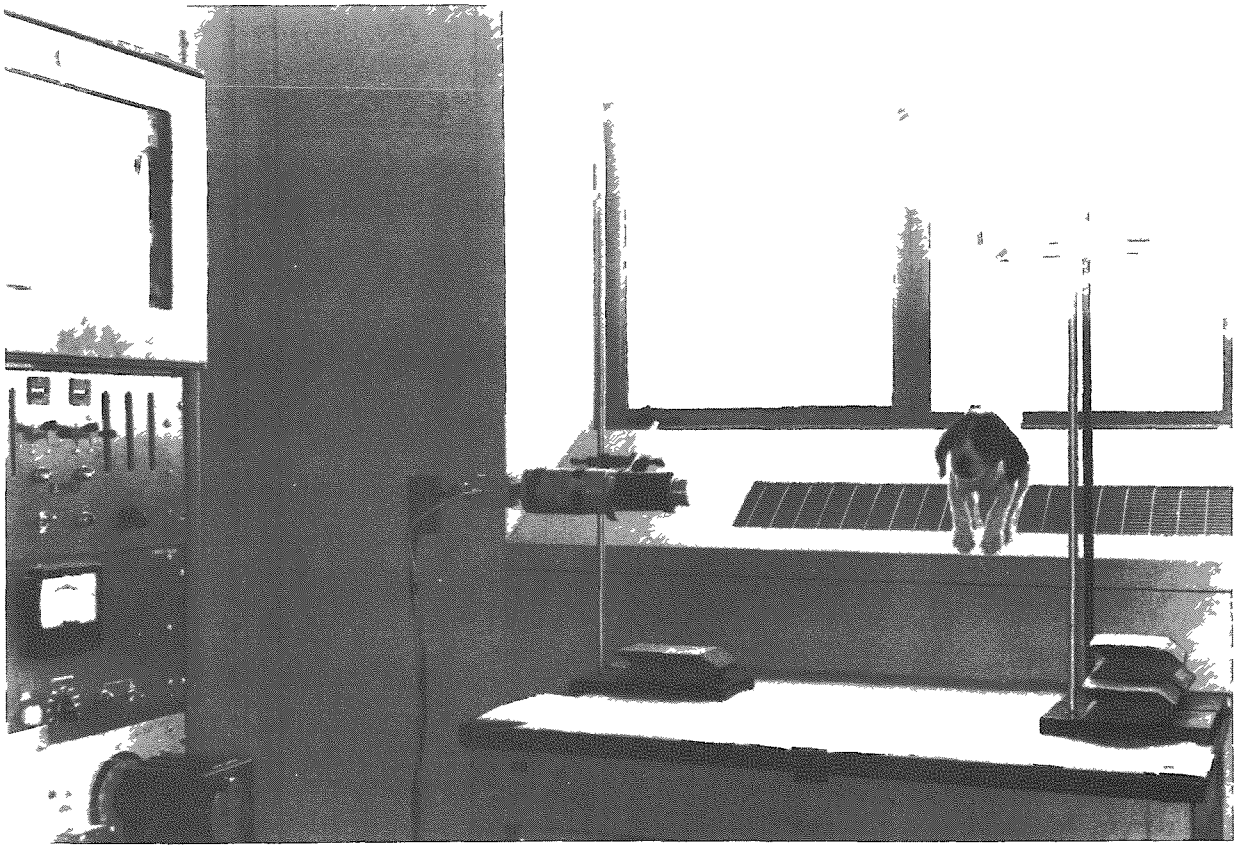

Figure 29. Experimental arrangement for in vivo gamma counting.

The relationships used to determine the whole-body retention of Sr^{85} from the in vivo gamma-ray activity are expressed by

$$R_X = \frac{OA_X}{OA_0} \cdot \frac{S_0}{S_X}$$

where R_X is the calculated retention at time t_X as a fraction of the injected dose, OA_X is the observed activity (cpm) in the dog at time t_X, S_X is the observed standard activity (cpm) at time t_X, S_0 is the observed standard activity (cpm) at time t_0, and OA_0 is the observed activity (cpm) in the dog immediately after injection. These relationships will have general application for long-term retention studies using in vivo measurements of gamma-emitting isotopes. The ratio (S_0/S_X) will correct the observed activity at any time, t_X, for physical decay, and for fluctuations in instrumental response from one time of observation to the next.

The present data have also been corrected for the effect of growth of the young dogs upon the geometrical relationship between the source and the detector. The procedure used to obtain this correction factor was briefly as follows.

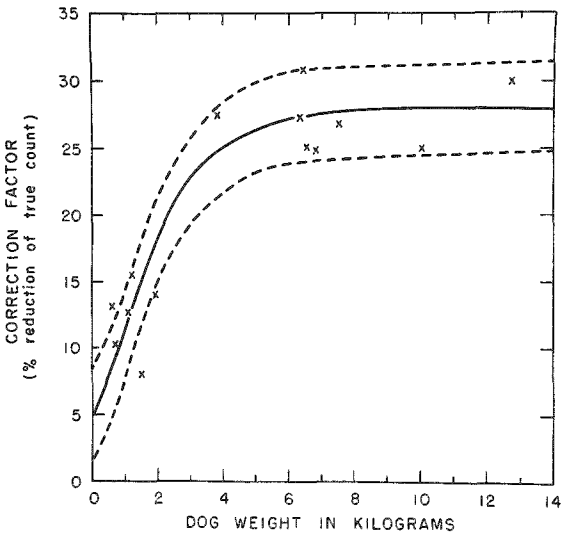

Figure 30. Correction curve for observed (true) counts. Solid line is the best visual fitting. The dashed lines represent the limits of the standard error.

The exact number of counts injected into the dog was determined from the difference in counts obtained from the injection syringe before and after injection of the isotope into the dog. The dog was then counted immediately after injection of the isotope. The difference between the syringe counts and the counts from the dog was then obtained. This difference is attributed jointly to gamma-ray absorption and variations in the geometrical relationships of source to detector. This factor is found to be relatively constant for an animal of a given size. Figure 30 is a plot of this factor for animals of various weights. The curve thus obtained was used as the dogs grew to correct the counts observed at various times after injection.

The curves in Figure 31 (visual fitting) are representative of retention of Sr^{85} in dogs of several different ages at time of injection. They indicate several points quite clearly. The fractional retention of strontium as a function of time after injection in dogs injected at 5 years of age may

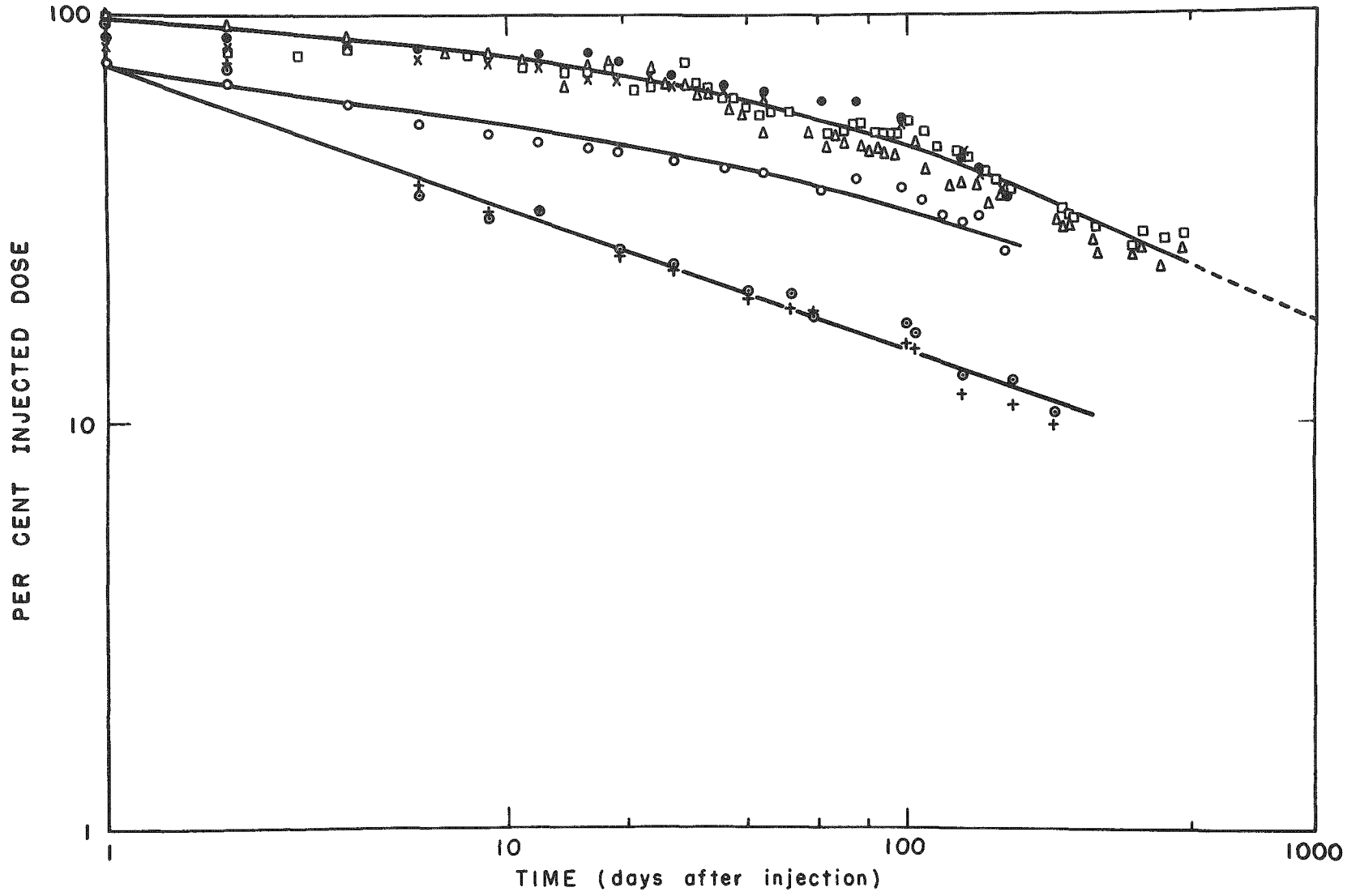

Figure 31. Retention of Sr^{85} in beagles. Each animal is designated by a different symbol. Upper curve, 4 dogs, 2 and 4 weeks old. Center curve, 1 dog 1 year old. Lower curve, 2 dogs, 5 years old.

be described by a straight line plotted on log-log paper over a period of about 200 days. The fractional retention of strontium in dogs injected as 2- to 8-week-old puppies is not a straight line on such a plot, and the curve for the dog one year old at injection is somewhat intermediate between these two extremes. The terminal slopes of the experimentally-determined lines appear to be nearly parallel. The implications of these findings for integrated dosage determinations and total skeletal turnover are being studied. We are at present analyzing the more complete data according to certain mathematical models. The advantages and disadvantages of each of these systems in interpreting the observed data will be considered.

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MICROINJECTION STUDIES ON AMOEBAE AFTER THEIR EXPOSURE TO A SUPRALETHAL DOSE OF X-RAYS OR γ-RAYS

Edward W. Daniels

The giant amoeba Pelomyxa illinoisensis survives for several days following a dose of ionizing radiation approximately equivalent to twice the lethal dose. Nonirradiated protoplasm from similar organisms, if injected into the irradiated amoebae by microfusion, will prevent death.[1] The most effective restorative components in the nonirradiated protoplasm have been found to migrate toward the heavy or centrifugal pole.[2,3] These include the nuclei, mitochondria, and pinocytosis vesicles (alpha granules).[4] Since it has proved difficult to achieve more complete isolation of these components within the living cell membrane, isolations from homogenate preparations have been attempted. In addition, some of the commercially available compounds found in subcellular fractions of other types of cells were obtained and injected by micropipette into lethally irradiated amoebae (Table 22).

It has been the aim, by using microfusion on the one hand and microinjection with micropipettes on the other, to find specific components capable of preventing radiation death, and thereby to learn more about the critical site of radiation damage within living cellular systems. All of the compounds and homogenate preparations were kept at 4° to 5° C to the time of injection into the amoebae. The quantity of fluid injected into each cell was not measured, but as an estimate, the average cell received about one part of fluid to 25 to 100 parts of amoeba protoplasm.

Injection Studies

Adenosinetriphosphate (ATP) had no beneficial effect on the irradiated amoebae whether injected alone or together with diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN), flavin adenine dinucleotide (FAD), coenzyme A (Co A), or cytochrome c. DPN, TPN, and FAD, compounds that are widely used in model systems of electron transport in the cell, were injected individually into different irradiated amoebae without positive restorative effects. Also, as shown in Table 22, commercial glucose-6-phosphate (G-6-P) was injected into lethally irradiated amoebae without positive results.

There is no evidence to indicate that the second phase of aerobic respiration, the Krebs cycle, is specifically impaired by radiation; if it is damaged, there is no restoration by the compounds injected. As shown in Table 22, both Co A and thioctic acid failed to aid recovery of the amoebae from radiation damage.

TABLE 22

Substances injected into supralethally irradiated amoebae (Pelomyxa illinoisensis). In no case were there any survivors after 10 days.

Substance injected	Medium	Concentration	Number of cells	Irradiation: 4000 r/min
Irradiated, non-injected controls	-	-	26	50 kr γ-rays
ATP	KK*	0.1 M	12	"
G-6-P	KK	0.01 M	40	30 kr X-rays
DPN	KK	0.1 M	20	50 kr γ-rays
DPN	KK	0.01 M	34	"
Irradiated, non-injected controls	-	-	20	50 kr γ-rays
TPN	KK + 0.001 M $MgCl_2$	0.1 M	16	"
TPN	"	0.01 M	12	"
FAD	"	0.1 M	16	"
FAD	"	0.01 M	6	"
Irradiated, non-injected controls	-	-	25	30 kr X-rays
Co A	KK + 0.001 M $MgCl_2$	0.001 M	31	"
DL-Thioctic (α-lipoic) acid	"	0.01 M	25	"
Cytochrome c	"	0.004 M	36	"
Irradiated, non-injected controls	-	-	50	30 kr X-rays
Thiamine-HCL RNA DNA	0.001 M $MgCl_2$ in glass-distilled water	0.05%	43	"
Irradiated, non-injected controls	-	-	50	30 kr X-rays
Yeast extract	KK	5%	27	"
Yeast extract	KK	1.5%	40	"
Chlorella cells	KK	About 12 per amoeba	16	"

* Kassel-Kopac salt medium.(10)

Although cocarboxylase, another coenzyme of the Krebs cycle, was not injected into the irradiated amoebae, a component of the cocarboxylase molecule, thiamine, was injected without any restorative effect.

According to Pace and McCashland[5] the cytochrome-cytochrome oxidase system is present in the closely related giant amoeba Pelomyxa carolinensis. On the assumption that a similar system is present in P. illinoisensis, commercially prepared cytochrome c from horse heart was injected into irradiated amoebae but failed to exhibit restorative properties (Table 22).

Recently, isologous ribonucleic acid (RNA)[6] as well as deoxyribonucleic acid (DNA)[7] have been shown to be of considerable therapeutic value in lethally irradiated rats. However, RNA injected together with thiamine and DNA was ineffective.

Yeast autolysate mixed with antigen and injected on the day following X-irradiation has been found to aid the restoration of antibody-forming capacity in rabbits.[8] A modification of this procedure was used on irradiated amoebae to test the capacity of the extract as a restorative agent (Table 22). Commercial powdered yeast extract in 5% solution was injected into irradiated cells within some 2 hr after exposure. This gave no therapeutic effect. A second solution (1.5%), allowed to stand for 12 hr before injection into lethally irradiated amoebae, was likewise ineffective. Jacobson also recently obtained negative results after treating the X-irradiated microorganism Chlamydomonas with yeast extract.[9]

To find out whether the protective effect is limited to living protoplasm of similar cells, foreign protoplasm from living plant cells (Chlorella) was also injected into lethally irradiated amoebae, but with negative results. However, because of micrurgical problems, the quantity injected was low (about a dozen Chlorella cells per amoeba).

Injection of Homogenate Fractions from Nonirradiated Isologous Amoebae

Since whole protoplasm as well as centrifuged portions of living cells produces recovery from lethal radiation injury,[1,2,3] further effort was made to fractionate the restorative protoplasm. Nonirradiated, noncentrifuged protoplasm was transferred directly by micropipette from the donor to the lethally irradiated recipient. This produced negative results, but the reason might be due to the low quantity of protoplasm transferred, about one part of donor protoplasm to approximately 100 parts of recipient protoplasm.

As another approach, nonirradiated amoebae were dried, then resuspended and injected into irradiated amoebae. Again, the negative result was possibly attributable to failure to transfer sufficient material. This approach

cannot be ruled out until the problems involving micrurgy and the proper handling of the dried protoplasm are worked out, and until a sufficient quantity of dried protoplasm is injected.

The main effort in using nonirradiated protoplasmic material from broken cells has been with the use of isologous homogenates. These were at first made in salt solutions,[5,10] then in sucrose solutions made up to 0.25 M with an intracellular salt medium.[10] The protoplasm had a tendency to clog the pipettes when salt media were used, particularly in the absence of sucrose. The sucrose, unfortunately, is toxic to the cells as shown by Kassel and Kopac.[10] The homogenates containing salt solution had to be either filtered or lightly centrifuged to eliminate coagula that otherwise clogged the micropipettes.

Apparently in one amoeba in 39, enough protoplasm was injected to bring about recovery. However, an attempt to repeat this experiment failed (Table 23). In later experiments, the homogenates were prepared in 0.25 M sucrose medium made with either salt solution or glass-distilled water. These homogenates were further fractionated by centrifugal force and cell fractions of a higher concentration were injected. The reasoning here was that a smaller, more concentrated inoculum should be sufficient, while at the same time less of the toxic sucrose would be injected. However, no further positive results were obtained from this approach.

In a second attempt with this method, only two centrifuged fractions were used, the nuclear fraction and the combined large-granule and small-granule fractions of mitochondria and morphological "microsomes." Some of the nuclei were left intact in the nuclear fraction, but, nevertheless, none of the lethally-irradiated amoebae injected with this material survived. After removal of the excess salt-sucrose fluid, the pellet of mitochondria and microsomes was stirred, and part of this material was drawn up into the pipette and injected into 35 lethally-irradiated amoebae (Table 23). None of these injected cells survived longer than the irradiated, noninjected controls.

Finally, a nonionizing medium other than sucrose was used: 0.25 M dextrose was made up with glass-distilled water at pH 7. Coagula were formed as before, and the homogenate had to be pulled through a sintered glass filter. After this step, the homogenate was injected without difficulty. However, this preparation also failed to prevent death or to prolong survival after irradiation.

TABLE 23

Homogenate fractions injected into supralethally irradiated (30 kr X-rays) amoebae (Pelomyxa illinoisensis).

Substance injected	Medium	Concentration	Number of cells*
Irradiated, noninjected controls	-	-	50
Direct transfer of nonirradiated protoplasm from donor cell by micropipette	-	-	34
Air-dried amoebae	0.001 M $MgCl_2$ in KK** medium	Suspension	13
Irradiated, noninjected controls	-	-	70
Homogenate	P C media[7]	1 part amoebae to 2 parts salt media	39†
Homogenate	"	"	24
Irradiated, noninjected controls	-	-	50
Homogenate of P. illinoisensis filtered through Ace Glass Co. medium sintered glass filter	KK	Estimated ratio Homogenate to amoeba 1:50	27
Irradiated, noninjected controls	-	-	75
Homogenate of P. illinoisensis centrifuged 5 min at 300 g, sediment discarded	KK	Estimated ratio Homogenate to amoeba 1:30	60
Irradiated, noninjected controls		-	50
Homogenate:			
Nuclear fraction (nuclei broken up); (700 g/10 min)	0.25 M Sucrose in KK	0.25 M	20
Mitochondrial fraction (8,500 g/10 min)	"	0.25 M	20
Microsomal fraction (18,000 g/1.5 hr)	"	0.25 M	36
Supernatant	"	0.25 M	42

*No survivors after 10 days, except as noted.
**Kassel-Kopac salt medium.[10]
† 3% survival after 10 days.

TABLE 23 (Cont'd.)

Substance injected	Medium	Concentration	Number of Cells*
Irradiated, noninjected controls	-	-	50
Homogenate filtered through sintered glass (Ace Glass Co.) medium filter	0.25 M Sucrose in KK medium	0.25 M	40
Nuclear fraction (nuclei intact)	"	0.25 M	20
Large- and small-granule fraction (18,000 g/7 min)	"	0.25 M	35
Irradiated, noninjected controls	-	-	75
Homogenate (one part amoebae and one part medium) passed through Ace Glass Co. medium filter	0.25 M Dextrose in glass-distilled water	0.25 M	55

*No survivors after 10 days.

Discussion

It appears from these studies that the protoplasmic systems which have the capacity to prevent radiation death in amoebae are extremely labile if released from the cell by homogenization or other means. All attempts to find a medium that will preserve the radiorestorative properties of nonirradiated protoplasm from broken amoeba cells have failed. Likewise, attempts to obtain cell restoration by injecting different commercially-prepared (heterologous) compounds and extracts have failed.

These results of the past several months' work give no encouragement for pursuing these approaches further. Emphasis is being returned to work with portions of living cells that still contain the various membranes intact. Some of these portions are injected by micrurgical methods into supralethally irradiated cells, while others are examined with the electron microscope. In this way new information should be obtained in regard to which components of living cells are most severely and critically damaged by radiation and what is specifically required to restore normal cellular function.

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PROGRESS REPORT: PLUTONIUM REMOVAL

III. Effect of Chelating Agents on the Ultrafiltration of Intravenously Administered Plutonium from Plasma

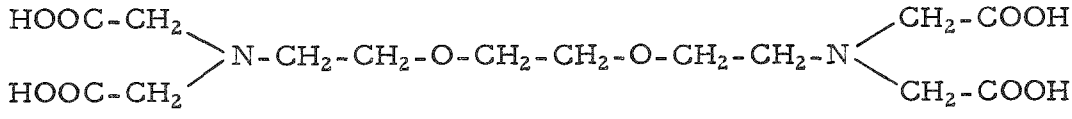
Arthur Lindenbaum, William M. Westfall, and Jack Schubert

Therapeutic experiments[1] involving the treatment of plutonium poisoning in rats have confirmed theoretical expectations suggesting the superiority of two new polyaminoacid-type chelating agents, diethylenetriaminepentaacetic acid (DTPA) and 2:2'-bis[di(carboxymethyl)amino] diethyl ether (BAETA) over ethylenediaminetetraacetic acid. (EDTA). The action of these chelating agents in the body is presumed to be due to partitioning of the colloidal plutonium as a result of competitive binding between tissue protein and chelating agent, thus allowing some of the plutonium to be excreted as a complex.

In order to demonstrate whether the above chelating agents do indeed exert their protective action in this way, an ultrafiltration method has been set up to measure the effect of type and concentration of chelating agent, as well as time elapsed after administration, on the ultrafilterability of intravenously administered plutonium citrate. For the purpose of such measurements some animals have received a therapeutically effective amount of chelating agent (calcium salt) 3 min following Pu administration, blood being removed after a 5-min or 30-min interval. In other experiments blood has been withdrawn from rats 30 min after Pu administration and the separated plasma mixed with amounts of chelating agent calculated to provide concentrations varying between 10^{-4} M and 10^{-8} M. The plasma in all cases has been placed in a Visking bag and centrifuged for approximately 20 min at 1000 g to produce a volume of ultrafiltrate sufficient for counting in a flow-type alpha counter.

EDTA and DTPA have so far been compared by both methods, and in each case the increased Pu filtration effected by DTPA closely parallels the increased excretion of Pu produced by DTPA in therepeutic experiments.

The ultrafiltration technique is also useful for screening new, potentially useful chelating agents. Thus the BAETA analog "Chel DE"* which differs from BAETA in having one more ethyl ether grouping,



* Obtained through the courtesy of the Geigy Industrial Chemicals Division of Geigy Chemical Corp., Yonkers, N. Y.

and might be expected to provide equal or enhanced protective action in vivo, is found to produce no significant increase in ultrafilterability beyond that produced by saline controls. Such an effect is possibly the result of a steric or a solubility effect, but in any case it would appear almost certain that this compound would produce no effect if used therapeutically against plutonium. Other ultrafiltration studies designed to screen other potentially useful chelating agents and to demonstrate the formation of filterable plutonium complexes in tissues, such as liver, bone and spleen, are in progress.

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ANTIBODY AGAINST THE TISSUE MAST CELLS

Yevette S. Lewis and Douglas E. Smith

Experiments are being carried out for the purpose of obtaining an antibody specifically against the tissue mast cells, which could be used to produce animals that are free of mast cells.

Rabbits were injected with whole mast cells derived from the peritoneal fluid of rats. Intravenous injections of 3 to 5 x 10^6 mast cells were administered over a 2-week period on days 1, 2, 3, 4, 5, 8, 9, 10, 12 and 14, and daily samples of serum were assayed thereafter for anti-mast-cell activity. This was determined by 1) ring precipitation tests to detect the presence of the antibody and 2) microscopic examinations of whole mounts of fixed and stained skin, mesentery and ear for mast-cell damage in rats injected intraperitoneally with immune serum. In vivo observations were also made on mesenteric mast cells bathed in normal and immune rabbit serum.

Within 3 to 5 days after the last inoculation, the presence of an antibody that destroyed mast cells became apparent in both ring tests and tissue preparations. The peak titer occurred between 10 and 12 days; observations on rats injected with antiserum showed that all of the mast cells in the mesentery and 50 to 100 per cent of the mast cells in the skin and ear were eliminated. The effects appeared to be restricted to mast cells; no change in other cell types were noted. Control rats showed no effects with normal rabbit serum.

In vivo observations of mesenteric mast cells exposed to antiserum revealed marked oscillations on the part of the otherwise motionless granules. At first only a few granules in the peripheral areas of the cell showed this movement but within about 10 min, all of the granules were involved. Much of this activity was accompanied by expulsion of granules from the cell and, in some instances, the entire cell appeared to rupture with release of granules into the tissue.

At present experiments are under way in an attempt to enhance the production of antibody and also to concentrate the anti-mast-cell fraction of serum.

AN IMPROVED METHOD FOR CONTRAST RADIOAUTOGRAPHY OF BONE

Phyllis E. Jenkins and William P. Norris

The technique of contrast radioautography aids greatly in the study of the uptake of radioisotopes by bone. For this type of study it is desirable to embed the tissue since embedding 1) reduces the probability of chemical artifacts, 2) increases resolution by minimizing radiation originating in the deeper layers of the tissue, and 3) facilitates handling the specimen.

The epoxy resins* possess certain favorable qualities as embedding media for bone tissue and have, therefore, been used extensively in this laboratory. These resins infiltrate the tissue adequately, polymerize rapidly with minimal shrinkage, are reasonably transparent, and machine excellently. They warp only slightly, if at all, even when stored over long periods of time; this allows reliable reproduction of the autograph upon successive exposures.

The excellent machining quality of the epoxy resins allows many variations in technique. Thus, it is possible to obtain not only smooth, flat surfaces on thick slabs of bone, but also thin ground sections, thin sections (50 to 100 μ) cut with a circular saw, and certain other variations. Indeed, it is possible with a heavy-duty microtome to remove very thin (6μ) sections from bone embedded in epoxy resins.

The chief disadvantages encountered in the use of epoxy resins derive from their amber color and their insolubility. Once embedded, the tissue is not readily cleared of the plastic. This imposes certain limitations - particularly in staining.

The procedure briefly outlined below, including time for polymerization, can be executed in three and one-half days. This is particularly important when dealing with short-lived isotopes.

Method

Cut the bone in the plane desired for observation using a motor-driven circular saw. The resulting slabs of bone are fixed and dehydrated in acetone and defatted in diethyl ether. Infiltrate the bone with epoxy resin, and allow it to polymerize in a suitable mold. Infiltration may be

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* Scotchcast Resin No. 2, Minnesota Mining and Manufacturing Co., was found to be suitable.

improved by using vacuum to remove entrapped bubbles of air, by using heat, and by the application of pressure during polymerization. Polymerization is complete after 4 to 6 hr at room temperature. The bone surface is exposed by machining and/or abrading.

The epoxy resins appear to be completely inert to photographic emulsions, and no image is produced by nonradioactive bones embedded in them. An example of a radioautograph made by this technique is shown in Figure 32, which illustrates the distribution of radium in the tibia of a dog.

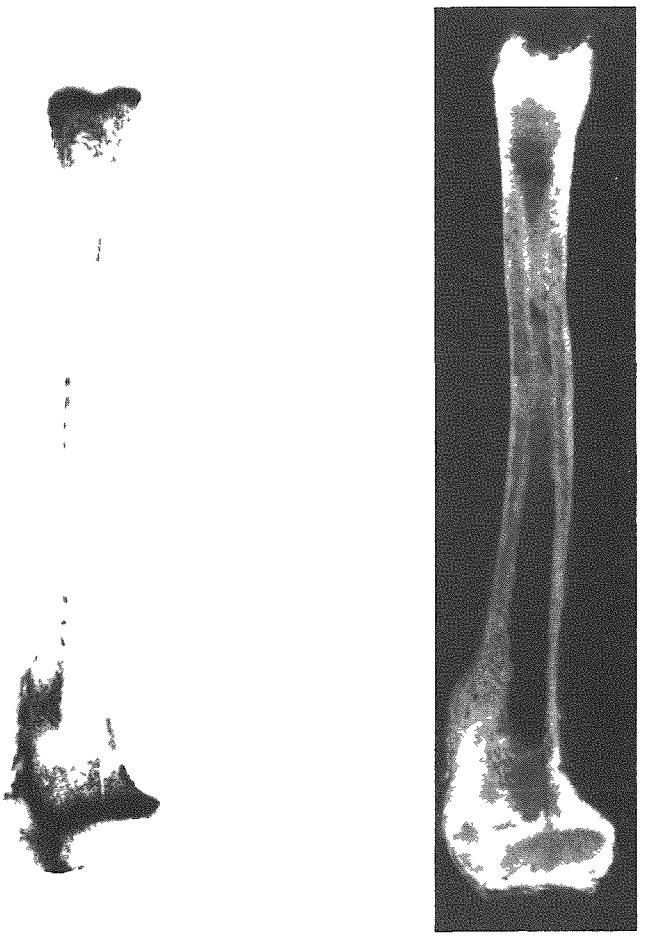


Figure 32. Right, photograph of dog tibia embedded in epoxy resin. Left, the corresponding radioautograph obtained on DuPont 506 X-ray film. The dog was injected with radium at the age of 248 days, and sacrificed 349 days after injection.

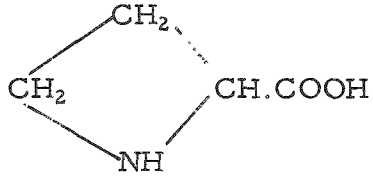
EXPERIMENTS ON THE BIOSYNTHESIS OF AZETIDINE-2-CARBOXYLIC ACID IN Convallaria majalis

F. Schlenk and J. L. Dainko

Azetidine-2-carboxylic acid was discovered independently by Fowden[1] and by Virtanen[2] in Liliaceae. In young leaves of Convallaria majalis (lily of the valley) as much as 70 per cent of the organic nitrogen fraction extractable by cold acid may consist of this compound. The structure, established by Fowden,[3] is represented by formula I.

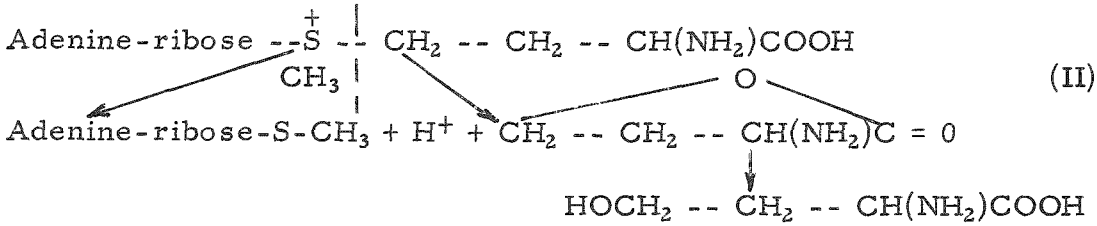
There have been several reports of experiments on the possible origin of this compound. Linko[4] observed no conversion of aspartic acid-C^{14} into azetidine-2-carboxylic acid as suggested first by Fowden.[3] More extensive experiments of Fowden and Bryant[5] showed that on exposure of the plant material to $C^{14}O_2$, the new cyclic imino acid, became radioactive more slowly than alanine, serine, and glutamic acid. The labeling was weak with C^{14}-aspartic or C^{14}-glutamic acid as precursors, and negative results were obtained with C^{14}-labeled glucose, γ-aminobutyric acid, or α, γ-diaminobutyric acid. The authors conclude[3] that the mechanism of synthesis of azetidine-2-carboxylic acid remains unsolved.

At about the same time, we were testing another hypothesis. It has been observed in this laboratory that S-adenosyl-L-methionine can undergo an enzymatic cleavage to L-α-amino-γ-butyrolactone and L-homoserine[6,7] as indicated in formula II. The frequent occurrence of homoserine in higher plants, including Liliaceae, has been demonstrated.[8] We assumed that under the influence of enzymes of Convallaria a split to a four-carbon fragment with ring closure to azetidine-2-carboxylic acid may occur (formula III). Likewise, it seemed desirable to test whether the sulfonium derivatives of methionine serve as precursors of homoserine.

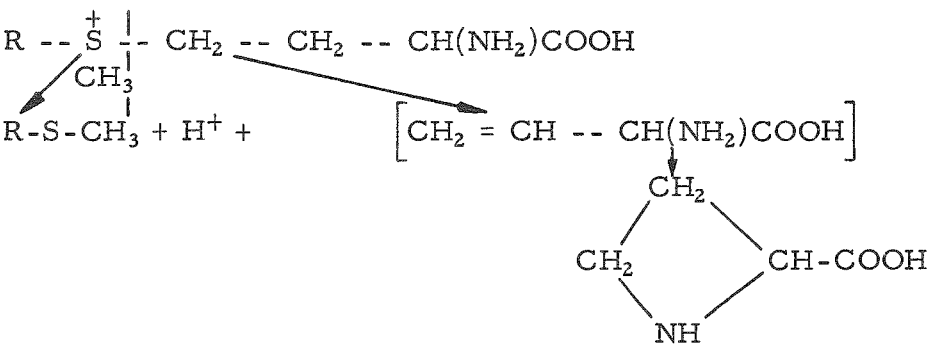


(I)

Azetidine-2-carboxylic acid



(II)



(III)

The following is a report on the effects of enzymes from Convallaria majalis on DL-methionine-2-C^{14}, S-methyl-DL-methionine-2-C^{14}, and S-adenosyl-L-methionine-2-C^{14}.

Experimental

Materials and Methods

Substrates. DL-Methionine-2-C^{14} was of commercial origin. It served for the synthesis of S-methyl-DL-methionine according to Toennies and Kolb,(9) and for biosynthesis of S-adenosyl-L-methionine by yeast under conditions which are selective for the L-stereoisomer.(10) A reference sample of azetidine-2-carboxylic acid was generously made available by Dr. F. C. Steward, Cornell University.

Plant material and enzymes. Young plants of Convallaria majalis were harvested in the spring. Enzyme extracts were prepared by grinding with 0.1 M phosphate buffer, pH 7.2, and sand, with or without subsequent removal of debris and dialysis. Pulp of leaves or roots prepared with a homogenizer was used in several experiments, and infiltration of substrates into whole leaves, exposed to an air draft for increased liquid uptake,(11) was also tried.

Analytical techniques. Paper chromatography was the principal method used to search for azetidine-2-carboxylic acid and homoserine. Butanol-acetic acid-water (12:3:5, vol/vol) and phenol-water (4:1, vol/vol) were selected as developers for two-dimensional migration. The former does not separate the two principal compounds, but it removes them from residual substrate; the latter affords satisfactory resolution of homoserine (R_f = 0.59) and azetidine-2-carboxylic acid (R_f = 0.77). Extracts from the incubated plant pulp samples were prepared as outlined by Fowden and Webb;(11) Dowex 50, H^+, was substituted for Zeocarb 215 since the latter was not available to us. It was ascertained that the first ammonia eluate from the column brought forth the bulk of radioactivity. The concentrated eluate was applied to Whatman No. 1 papers, and, after development, the

papers were scanned with a mechanical recording gas-flow counter developed in this Laboratory;[12] eluates from the papers were counted in the Packard Tricarb Liquid Scintillation Counter.

Incubation of Sulfonium Compounds with Cell-free Extracts of Convallaria majalis

A series of experiments was carried out in which S-adenosyl-L-methionine or S-methyl-L-methionine was incubated with dialyzed Convallaria extract at 35° for intervals ranging from 2 to 24 hr; the concentrations in the incubate were 5.0μ M of substrate per ml, 1 to 4 mg of soluble protein from dialyzed plant extract, 0.01 M phosphate at pH 7.4. Controls without enzyme were included in each experiment to account for the spontaneous decomposition of S-adenosylmethionine.

The solutions were surveyed by paper chromatography in a search for the characteristic brown spot resulting from the treatment of azetidine-2-carboxylic acid with the ninhydrin reagent. No evidence of the formation of azetidine-2-carboxylic acid was found although the sensitivity of the tests would have permitted the detection of 5 per cent conversion of the substrate to this compound. It appeared possible that the products formed from the methionine sulfonium compounds might undergo rapid further metabolism. Control experiments were carried out, therefore, to test the stability of the surmised end products by exposing substrate concentrations of them to the plant extracts. While azetidine-2-carboxylic acid and homoserine were found to be stable, rapid splitting of 5'-methylthioadenosine to 5-methylthioriboside and adenine was observed. This indicated the presence of a nucleosidase identical with or similar to that discovered recently by Shapiro and Mather[7] in extracts of Aerobacter aerogenes; its properties will be described in a separate report.

The failure to observe any relation between the sulfonium compounds and azetidine-2-carboxylic acid suggested tracer experiments which would increase the analytical sensitivity.

Tracer Experiments

The following substrates were used: DL-methionine-2-C^{14}, activity 5.5×10^5 cpm per μM; S-methyl-DL-methionine-2-C^{14}, 6.7×10^4 cpm per μM; and S-adenosyl-L-methionine-2-C^{14}, 9.5×10^3 cpm per μM.

In one experiment, stems of young leaves (weight 2.3 to 2.7 g) were placed in 1.0 ml of methionine (13.4 μM), 1.0 ml of S-methylmethionine (13.7 μM), and 1.0 ml of S-adenosylmethionine (27.5 μM). Details of the experiment were those suggested by Fowden and Bryant [5] The material was pulped after 6 hr at room temperature and extracted with 70 per cent alcohol; the extract was purified by an ion exchange column, and the first eluate with ammonia, which contained the bulk of radioactivity, was evaporated and taken up in a minimal amount of water as outlined by Fowden and Webb.[11]

In other experiments, the above-ground parts and the root systems of young plants were homogenized separately in equal volumes of phosphate buffer, 0.5 M, pH 7.2. The pulp was incubated for 6 hr and 45 min at 30° with an equal volume of the substrates listed above.

Two-dimensional paper chromatography of aliquots of the extracts obtained in these experiments was carried out. The location of the components by ultraviolet scanning and ninhydrin spray offered no difficulty. Homoserine and azetidine-2-carboxylic acid are prevalent constituents of Convallaria extracts and, in case of doubt, carrier material was added to facilitate identification of the spot. Radioscanning of one- and two-dimensional paper chromatograms showed the bulk of substrate to be unaltered. Considerable activity was found in the homoserine spots, but azetidine-2-carboxylic acid was essentially inactive. The lower limit of detectability of conversion of the substrates into the cyclic amino acid would have been 0.1 per cent with methionine, 0.5 per cent with S-methylmethionine, and 4.8 per cent with S-adenosyl-L-methionine. To increase the sensitivity of testing in the last-named instance, the paper spots of azetidine-2-carboxylic acid were eluted, and the radioactivity in the eluates was determined by scintillation counting. This increases the sensitivity of the test about tenfold and insured that less than 0.5 per cent, if any, conversion of S-adenosyl-L-methionine to azetidine-2-carboxylic acid had occurred.

Conclusion and Summary

From the present experiments it appears that S-adenosylmethionine and S-methylmethionine do not play a major role in the formation of azetidine-2-carboxylic acid by Convallaria majalis. The failure to demonstrate this process in vitro does not strictly exclude its occurrence in vivo. Reference should be made to the discussion of azetidine-2-carboxylic acid biosynthesis by Fowden and Bryant;[5] their appraisal of the difficulties of studying the process applies fully to the present work.

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