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PROGRESS REPORT: DISEASES OF LABORATORY ANIMALS

Notoedres muris Infection of Rats

Robert J. Flynn

Notoedres muris causes a scabies-like mange of rats characterized by the occurrence of red papules and thick, horny crusts on the hairless or sparsely haired parts of the body. The ears, nose, and tail are the sites usually affected; however, in extreme cases, the feet and external genitalia may become involved.(1)

The opportunity to observe this infection first-hand and to test the effectiveness of the acaricidal treatment previously developed for the control of ectoparasites of mice (2) occurred when five commercially-bred laboratory rats with "tumors on their ears" were forwarded to the author for diagnosis.

Description

The ears of all five rats appeared macroscopically to be covered with "papillomas" or "tumors" (Figure 1A). The lesions of the nose appeared as wart-like, horny projections; those of the tail, as red vesicles or papules. Through a dissecting microscope, the tumor-like lesions of the ears appeared as yellowish, keratinous crusts. Breeding tunnels, containing countless specimens of Notoedres muris (Figure 1B) in all stages of development, were found under these crusts.

Treatment

Two of the rats were given three weekly treatments using a 2% aqueous suspension of a wettable powder containing 15% Aramite [2-(p-tert-butylphenoxy) isopropyl-2-chloroethyl sulfite]. Two rats were not treated.

Results

By the 30th day after the initiation of treatment, the two treated rats were completely free of lesions, and no mites were demonstrable (Figure 1C). The two untreated rats remained unchanged.

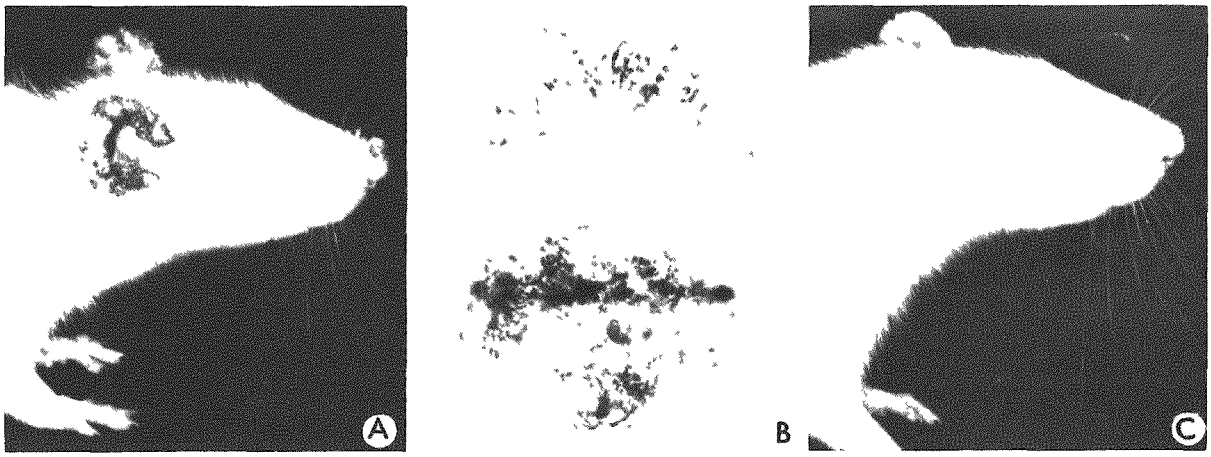

Figure 1

A, ear mange of rat, untreated; B, etiological agent: Notoedres muris (female); C, same rat as in A, 30 days after treatment.

References

1. LaPage, Geoffrey. Veterinary Parasitology. Springfield, Illinois: Charles C. Thomas, 1956, pp. 705-708.

2. Flynn, R. J. Control of ectoparasites in mice. Proc. Animal Care Panel 6 75-91 (1955).

PROGRESS REPORT: DISEASES OF LABORATORY ANIMALS

Failure of Stylomycin to Eliminate Syphacia obvelata Infection

Robert J. Flynn and LeRoy O. Bibbs

A mouse breeding colony, maintained at this Laboratory and initiated by foster nursing caesarian-born CF No. 1* mice on CFN* rats, was found to be infected with Syphacia obvelata. It has been reported(1) that the addition of Stylomycin** to the drinking water in single doses of 0.62 mg or higher per mouse was more than 99.5% effective in removing these pinworms. Therapy with Stylomycin was therefore attempted.

Methods

The entire colony was treated: thirty breeding groups each consisting of 12 adult females, 2 adult males, and varying numbers of suckling mice. Three days a week, for four weeks, each group was given as its sole drinking fluid, 250 ml of water containing 31.25 mg Stylomycin (equivalent to 17.9 ml of water and 2.23 mg of Stylomycin per adult mouse). To increase palatibility, 5 ml of Coke† syrup was added per 100 ml of medicated solution.

Each breeding group was housed separately in a "shoe-box" type cage consisting of a stainless steel top with built-in feeder and bottle holder, and a plastic bottom.(2) Autoclaved white pine shavings were used as bedding and Wayne Lab-Blox as the diet.

At least once a week, all cage bottoms and tops, water bottles, nipples, and stoppers were washed with hot detergent solution and rinsed with 180-210°F clear water.

Previously sterilized cages with wire-mesh floors and stainless steel pans were used to collect fecal samples for examination. The mice, by breeding groups, were housed in these cages for 24 hours; the composite fecal samples recovered were examined by the flotation method.(3)

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*Carworth Farms, New City, New York.

**Puromycin HCl, 6-dimethylamino-9-(3'-p-methoxy-L-phenylalanylamino-3'-deoxy-D-riboxyl)-purin. American Cyanamid Co.

†Coca-Cola Company

Results and Discussion

Stylomycin failed to eliminate the infection. Before treatment, 70% of the breeding groups were positive for S. obvelata; four weeks after the last treatment, 63% were positive.

The possibility that the unsterilized, unpasteurized commercial feed might have been responsible for the failure was ruled out when it was observed that an adjacent mouse colony, after being fed the identical food for over 6 months, remained completely negative for S. obvelata.

Since the life cycle of this parasite is 12-15 days,[4] the procedures followed should have eliminated the infection completely. Either the drug used is not as effective as the earlier work indicated, or some unknown "break" in technique occurred. The latter possibility is being investigated further.

References

1. Habermann, R. T. and F. P. Williams. The efficacy of some piperazine compounds and Stylomycin in drinking water for the removal of oxyurids from mice and rats and a method of critical testing of anthelmintics. Proc. Animal Care Panel 7 89-97 (1957)

2. Flynn, R. J. Small animal cages. Proc. Animal Care Panel 4 186-188 (1953).

3. Coffin, D. L. Manual of Veterinary Clinical Pathology. Ithaca, New York: Cornell University Press, 1953.

4. Chan, K. F. Life cycle studies on the nematode Syphacia obvelata. Am. J. Hyg. 56 14-21 (1952).

RED-FAR RED INTERACTION IN OXIDATIVE PHOSPHORYLATION

S. A. Gordon and K. Surrey

Radiation in a broad region about 1-μ wavelength (near infrared) will increase the number of chromosomal aberrations induced by X-rays. This phenomenon has been shown to occur in the animal[1] and in the plant.[2] It has also been used to enhance the effect of X-rays on tumor regression.[3] Subsequently, Withrow and Moh[4] demonstrated that far-red radiation between 700 to 800 mμ, and not the infrared, was the active spectral region. Moreover, the potentiation of X-ray-induced chromosomal aberrations by far-red light could be nullified by concomitant or subsequent irradiation with red light.[5] This reversal suggests that the photoreceptor of the visible radiation is analogous, if not identical, to that functioning in plant photomorphogenesis.[6]

Recent studies of Wolff and Luippold[7,8,9] indicate that the recombination following X-ray-induced chromosomal breaks is dependent upon oxidative metabolism. The energy requirement for recombination can be supplied through, and probably normally comes from, the generation of adenosinetriphosphate (ATP). Rejoining is impeded by post-irradiation anoxia, low temperature, and metal poisons - known respiratory depressants. It is also impaired by the respiratory uncoupler, dinitrophenol. Supplying ATP increases the frequency of restitution.

The above two groups of work in conjunction suggest that the biochemical basis of the far-red potentiation of chromosomal breaks is the impairment of ATP generation. This hypothesis has been examined experimentally with both plant and animal tissues.

Experimental

Avena seedlings were grown in the dark for $1\frac{3}{4}$ days. They were then irradiated for 3 hr under the biological spectrograph[10] at the 650- and 725-mμ loci. Incident energies were adjusted to give a flux at the red and far-red positions of about 20 ergs/mm^2/sec. After 20 hr the shoots were cut at the scutellae. The mitochondrial fraction was isolated and assayed for ATP-producing capacity. Table 1 indicates that the far-red significantly reduced, and the red significantly enhanced, the phosphorylative capacity of the mitochondria.

The possibility was considered that the differences shown in Table 1 were caused by differences in mitochondrial concentration, mass, or volume induced by previous irradiation of the plant. This seems unlikely: no significant correlated differences were observed in the N

precipitable by trichloroacetic acid, in the mitochondrial packed volume (hematocrit capillary method), or in the mitochondrial number (counts of evaporated aliquots with the electron microscope) of the mitochondrial suspensions.

TABLE 1

Phosphorylation rates of mitochondria isolated from oat seedlings previously irradiated by red or far-red light.

	$\underline{b} \pm \sigma_m$ (30°C)* n = 6
Red	1.03 ± 0.01
Dark	0.87 ± 0.01
Far-red	0.71 ± 0.02

*$\underline{b}$ represents the average μ moles phosphate esterified per ml enzyme per minute, as minimized by least squares, for the linear period of activity.

No significant difference in phosphorylative capacity was found after directly irradiating isolated Avena mitochondria with the red or far-red light. Though this suggests that the mitochondrial response in the plant could be indirect, a direct radiation effect on mitochondria of the rat liver was observed. Table 2 indicates that far-red light causes a significant decrease in the rate of ATP generation, compared to the red, when liver-mitochondria are added to a phosphorylation medium and then irradiated. In the experiment represented at the left of Table 2, esterification took place at 22°C. At the right is shown the turnover when the mitochondrial suspension and medium was maintained at 3°C during irradiation.

Mitochondrial suspensions alone were then irradiated at 3°C. Periodically, aliquots were removed for assay of activity at 30°C. These activities, expressed as successive rate constants, are given in Figure 2. The change in rate constant as a function of time in the dark and under red or far-red light is given in Figure 3 as a semilog plot. These curves indicate that the zero-order phosphorylation rates decay exponentially in the dark. The decay constant is increased by a factor of about $1\frac{1}{2}$ under far-red and decreased by a factor of about $\frac{1}{3}$ under red irradiation.

TABLE 2

Phosphorylation rates of rat liver mitochondria at 22° and 3° C while under continuous irradiation by red and far-red light.

	22° C $\underline{b} \pm \sigma_m$* (n = 6)	3° C $\underline{b} \pm \sigma_m$* (n = 12)
Red	0.60 ± 0.003	0.029 ± 0.002
Dark	0.57 ± 0.03	0.013 ± 0.001
Far-red	0.45 ± 0.01	0.018 ± 0.003

*$\underline{b}$ represents the average μ moles phosphate esterified per ml enzyme per minute, as minimized by least squares, for the linear period of activity.

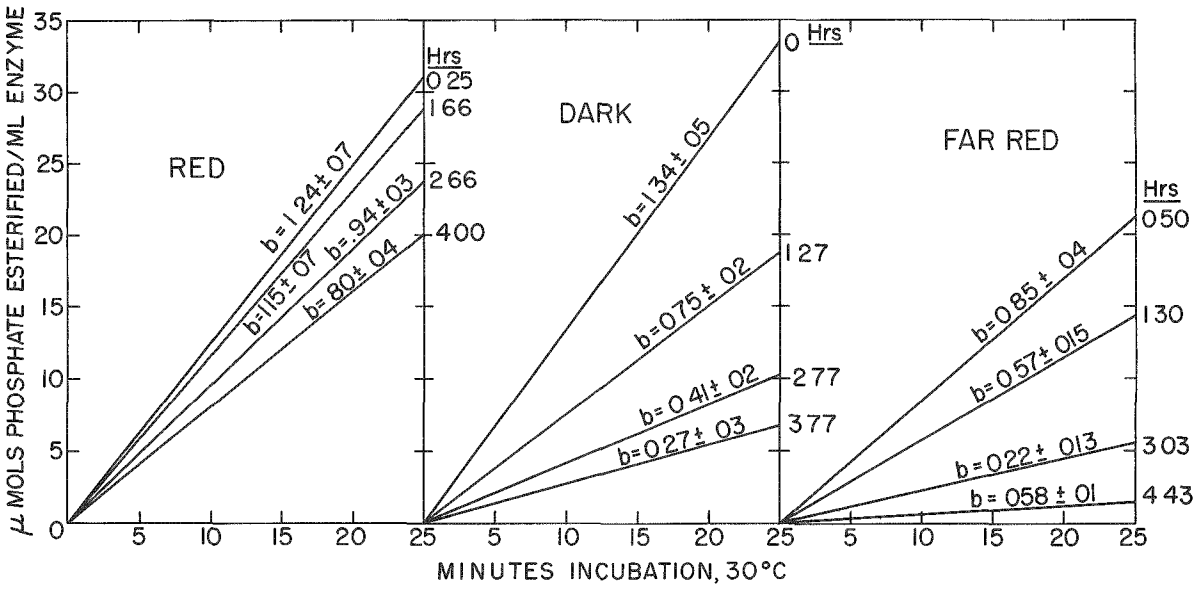


Figure 2

The effect of pre-irradiation with red and far-red light on the phosphorylative activity of rat-liver mitochondria. The mitochondria were kept at 3° C during the irradiation periods, whose duration is indicated under "Hrs."

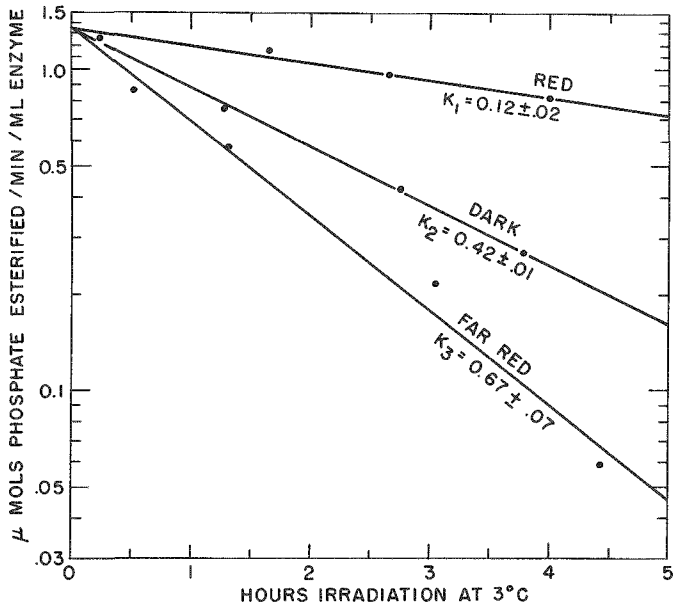


Figure 3

The effect of red and far-red light on the decay of mitochondrial phosphorylative capacity.

The effect of far-red radiation apparently can be nullified by the red in an alternating sequence. A mitochondrial suspension was placed under far-red for slightly less than 1 hour and then aliquots were taken for assay. A portion of the suspension was then moved to the red locus of the spectrograph, allowed to remain for ca. an hour, and aliquots were again taken for assay. A portion was then returned to the far-red locus. The results, shown in Figure 4, indicate that at least two successive reversals with red and far-red are possible.

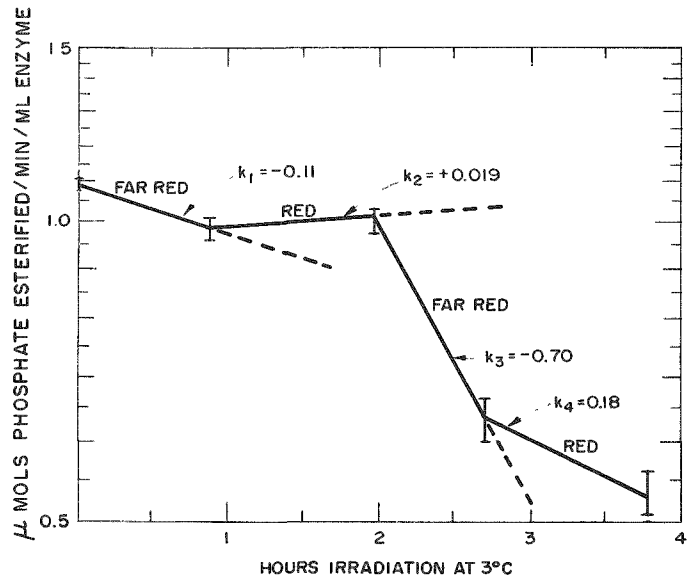


Figure 4

Phosphorylative capacity of rat-liver mitochondria at 30° C as affected by alternating pre-irradiation with far-red and red light.

Discussion

It may be concluded that ATP generated by mitochondrial phosphorylation is significantly affected by red and far-red radiation. Red and far-red action has been demonstrated chiefly at the morphological and cytological levels by previous work. The present experiments have shown its function at the biochemical level. As far as we are aware, this is the first demonstration of a red-far red reaction and its reversibility for animal tissues.

If chromatid reconstitution requires ATP, the impairment of ATP generation appears to be a demonstratable and adequate biochemical basis for the far-red potentiation of X-ray-induced chromatid aberrations.

These experiments indicate that the primary locus of action of the red spectrum on the chromosome might be a cytoplasmic organelle other than the nucleus. Not precluded, however, is an analogous phenomenon in the nucleus.

Finally, this work suggests the possibility that the rate of oxidative phosphorylation may play the determinative role in the morphogenic responses to the red end of the visible spectrum.

References

1. Kaufmann, B. P., A. Hollaender, and H. Gay Modification of the frequency of chromosomal rearrangements induced by X-rays in Drosophila. I. Use of near infrared radiation. Genetics 31 349-367 (1946).

2. Swanson, C. P. and A. Hollaender. The frequency of X-ray-induced chromatid breaks in Tradescantia as modified by near infrared radiation. Proc. Natl. Acad. Sci. U.S. 32 295-302 (1946).

3. Clark, G. M. Effects of infrared and X-radiation on a transplantable tumor of mice. J. Heredity 47 188-192 (1956).

4. Withrow, R. B. and C. C. Moh Nonionizing radiant energy as an agent in altering the incidence of X-ray-induced chromatid aberrations. I. Effects of Far-red and Infrared radiant energy on Tradescantia and Vicia. Radiation Research 6 491-500 (1957).

5. Moh, C. C. and R. B. Withrow. II. Reversal of the far-red potentiating effect in Vicia by red radiant energy. Radiation Research 10 13-19 (1959).

6. Hendricks, S. and H. A. Borthwick. Photoperiodism in plants. Proc. First Int. Photobiol. Congr., Amsterdam, 1954. Wageningen: H. Veenman and Sons. pp. 23-35.

7. Wolff, S. and H. E. Luippold. Metabolism and chromosome break rejoining. Science 122 231-232 (1955).

8. Wolff, S. and H. E. Luippold. The biochemical aspects of chromosome rejoining. In Progress in Radiobiology, Edinburgh: Oliver and Boyd, 1956, pp. 463-470.

9. Wolff, S. and H. E. Luippold. Modification of chromosomal aberration yield by post-irradiation treatment. Genetics 43 493-501 (1958).

10. Monk, G. S. and C. F. Ehret. Design and performance of a biological spectrograph. Radiation Research 5 88-106 (1956).

IN VIVO MEASUREMENT OF Sr^{90} IN DOGS

Philip F. Gustafson and Patricia J. Bergstrand

In November 1955 a group of 14 dogs that had been injected 1 to $9\frac{1}{2}$ years previously with Sr^{90} was measured by bremsstrahlung counting with an NaI scintillation counter.(1) The purpose of the study was to investigate the possibility of using this method of in vivo counting of Sr^{90} to determine the body burden of this radioelement. The success and further application of the technique is illustrated by reports of similar procedures used elsewhere for Sr^{90} determination.(2,3) Of the 14 dogs originally measured, 5 are still alive and have been counted again within the past few months.

The counting of these and the other dogs reported here was done in the steel room of the Biology Division using a 5 x 4 in. NaI crystal as the photon detector. A lucite β-ray absorber, $\frac{1}{2}$ in. thick, surrounded the crystal. The dogs were placed in a standing position in a plastic sling, which held them comfortably without permitting much movement. The distance from the center of the crystal to the backbone of the dog was maintained at 50 cm. Data were collected on an Argonne Type 256-channel analyzer, the spectral region extending from 20 to 200 kev.

The amounts of Sr^{90} present in the experimental dogs were obtained by comparision with two dogs counted shortly after they had been injected with known amounts of Sr^{90}. The use of two "standard dogs" of different weights (4.6 kg and 12.6 kg) made it possible to find the variation of counting rate per μc of Sr^{90} body burden as a function of body size and/or body weight. A third dog weighing 1 to 2 kg will also be injected so that the calibration may be extended to smaller dogs. The background counting rate over the spectral range of interest was of the order of 540 cpm; one μc of Sr^{90}-Y^{90} gave 100 and 160 cpm in vivo in the 12.6- and 4.6-kg animals, respectively.

New measurements of 4 of the dogs extend retention values for Sr^{90} to 11 to $12\frac{1}{2}$ years after injection. In addition, the one surviving high-level dog counted in 1955 was also recounted. Table 3 indicates the current retention values along with those found in 1955. The retentions to be expected from the 1955 data according to the power function described by Stover et al.(4) ($R_t = 0.7\ t^{-0.2}$) are also shown for comparison. The data agree fairly well with this form of the power function.

In 1956 the Pathology Group undertook a study of Sr^{90} in very young beagles. Three pregnant dogs were injected from 1 to 8 days before parturition, and the young received no further Sr^{90} except for that derived from suckling. The initial injected dose and the retention values for the three dams are indicated in Table 4. Retention in each dog in the three litters

TABLE 3

Sr^{90} body burdens as measured by bremsstrahlung method

Dog	Injected dose, mc	Age at injection	Retention 1955, μc	Time since injection, years	Retention 1958, μc	Time since injection, years	Retention 1958 calculated by $R_t=0.7t^{-0.2}$, μc
QA135	1.010*	6 mo	24.9	9.5	24.0	12	22.2
QA147	1.070*	Adult	9.9	9.5	8.4	12.5	8.6
QA181	0.086	5 mo	20.7	8.5	19.5	11	18.4
QA182	0.094	5 mo	14.4	8.5	14.9	11	12.8
QA184	2.25	6 yr	266.8	1.0	183.5	3.5	194.2

*Unknown fraction of Sr^{89} present in injection solution.

TABLE 4

Retention of Sr^{90} in female beagles

Dog	Age at injection, year	Injected dose, μc	Time after injection, year	Retention	
				μc	%
PW81	4	98	$1\frac{1}{2}$	6.7	6.8
PW82	4	95	$1\frac{1}{2}$	5.3	5.6
PW83	4	904	$1\frac{1}{2}$	36.4	4.0

at the end of 2 years amounts to 1 to 2% of the dose given to the mother (Table 5). Expressed in these terms, retention appears to be low, but it should be pointed out that the initial dose present in the newborn pups, as measured with a somewhat different counting procedure, indicates that retention two years after injection is of the order of 90%. Thus it is that amount of Sr^{90} crossing the placental barrier is small, but the retention is high.

An attempt was made to incorporate levels of 150 μc/kg and 15 μc/kg of Sr^{90}, which are comparable to that administered to QA184 and its litter mates and to QA181 and its litter mates, respectively (see Table 3). This was done by the systematic injection (1 injection each working day) of each animal of subsequent litters from each of the 3 females. The term of injection was not complete at the time of measurement in all cases, but the total amounts injected and the amounts retained are tabulated in Table 6.

TABLE 5

Sr^{90} body burden in beagles injected prenatally

Dog	Time after injection, year	Retention, μc	Body burden,* %
Litter PW81-L_1**			
PW81-L_1-2	2	1.6	1.6
PW81-L_1-3	2	1.5	1.5
PW81-L_1-4	2	1.5	1.5
PW81-L_1-5	2	1.8	1.8
Litter PW82-L_1**			
PW82-L_1-1	$1\frac{1}{2}$	2.2	2.3
PW82-L_1-2	$1\frac{1}{2}$	2.2	2.3
PW82-L_1-3	$1\frac{1}{2}$	1.8	1.9
PW82-L_1-5	$1\frac{1}{2}$	2.0	2.1
Litter PW83-L_1**			
PW83-L_1-1	2	11.2	1.2
PW83-L_1-3	2	16.6	1.8
PW83-L_1-4	2	13.7	1.5
PW83-L_1-5	2	7.3	0.8
PW83-L_1-6	2	10.5	1.2
PW83-L_1-7	2	13.3	1.5
PW83-L_1-9	2	11.5	1.3

*Expressed as percent of dose administered to dam.

**Litter PW81-L_1: 98 μc Sr^{90} was injected into the dam 6 days before parturition; litter PW82-L_1: 95 μc was injected into the dam 1 day before parturition; litter PW83-L_1: 904 μc was injected into the dam 8 days before parturition.

TABLE 6

Summary of periodically injected beagles

Dog	Injected dose, c	Retention, μc	Retention, % injected dose
Litter PW81-L_2*			
PW81-L_2-6	1310	452.6	34.5
PW81-L_2-7	"	491.1	37.5
PW81-L_2-9	"	552.0	42.1
PW81-L_2-10	"	513.7	39.2
PW81-L_2-11	"	492.5	37.6
Litter PW82-L_3*			
PW82-L_3-15	126.4	62.6	49.5
PW82-L_3-16	"	58.0	45.9
PW82-L_3-17	"	51.5	40.7
PW82-L_3-18	"	59.1	46.7
PW82-L_3-20	"	65.3	51.7
Litter PW83-L_2*			
PW83-L_2-12	522	139.4	26.7
PW83-L_2-13	"	158.0	30.3
PW83-L_2-14	"	174.0	33.3
PW83-L_2-15	"	156.5	30.0
PW83-L_2-16	"	194.9	37.3
PW83-L_2-17	"	164.7	31.5

*Litter PW81-L_2 was born July 16,1957, and has had 226 injections of 5.8 μc/injection, to date. Litter PW82-L_3 was born November 28, 1957, and has had 218 injections of 0.58 μc/injection, to date. Litter PW83-L_2 was born July 20, 1957, and has had 90 injections of 5.8 μc/injection. The last injection was made November 27, 1957; the injections were then stopped because of multiple fractures among the animals in this litter.

It is apparently true that retention is a function of age and moves in the direction of higher retention in younger animals. This is, in fact, illustrated in the case of the pups born shortly after injection of the dam, where the retention does not follow $R_t = 0.7\ t^{-0.2}$, but corresponds to a case where the coefficient is larger and the exponent either the same or somewhat smaller. It is proposed to investigate more thoroughly the nature of retention in very young dogs and to attempt to build a model to fit the retention pattern exhibited in the sequential injection cases. It is also contemplated that all these dogs will be counted periodically.

References

1. P. F. Gustafson and C. E. Miller. Radium Toxicity. Bremsstrahlung measurement of Sr^{90} in dogs. Report on Biological Medical and Biophysics Program. Argonne National Laboratory, ANL-5518, 1956. pp. 49-51.

2. C. W. Mays. Bremsstrahlung counting of Sr^{90} injected dogs. Paper presented at the Third Annual Meeting of the Health Physics Society, Univ. of Calif., Berkeley, June 9-11, 1958.

3. C. W. Mays, D. H. Taysum and W. Fisher. Radiobiological Laboratory Progress Report. COO-215 1958. pp. 139-146.

4. B. J. Stover and D. R. Atherton. Sr^{90} metabolism in dogs. Proc. Soc. Exptl. Biol. Med. 99 201 205 (1958).

CONSEQUENCES OF THE CONTINUOUS INGESTION OF Sr^{90} BY MICE

I. Experimental Design and Results up to 200 Days

Miriam P. Finkel, Patricia J. Bergstrand, and Birute O. Biskis

Human contamination with Sr^{90} occurs primarily through the ingestion of foods containing Sr^{90}. Although animal experiments involving the injection of solutions of radiostrontium provide important information bearing on the hazards of Sr^{90} contamination, they differ from continuous ingestion in two important respects. First, the distribution of the isotope in the skeleton is very uneven if the total amount is given in a single injection, and it becomes more uniform as the number of fractionated injections increases. The greatest uniformity would be expected to follow a continuous feeding regimen. Second, Sr^{90} is usually injected in equilibrium with its Y^{90} daughter, which follows the distribution pattern of yttrium and contributes significantly to the over-all irradiation in the early period after administration. On the other hand, the absorption of ingested yttrium is negligible, so when it enters the body with food, it irradiates the intestinal tract primarily. For these reasons, and because it is desirable to study the effects of continuous internal irradiation in very young animals, the following experiment was undertaken.

Materials and Methods

Food preparation. Sr^{90} at the six levels listed in Table 7 is incorporated in milled mouse food before pelleting. The highest level is equivalent

TABLE 7

Reproductive history

Level	$\mu c\ Sr^{90}$ / g Ca	No. of females	Litter production			Total No. of young / Total No. of litters	
			%	%/♂-day*	nonviable	Alive at birth	Females alive at 35 days
1	10.0	230	17.8	5.9	2.4	8.1	2.3
2	5.0	236	8.9	4.5	4.8	7.9	2.5
3	2.5	285	16.2	8.1	4.4	8.3	2.8
4	1.0	293	6.8	6.8	10.0	7.6	2.5
5	0.1	332	12.1	12.1	5.0	8.4	2.3
6	0.01	317	12.0	12.0	13.1	7.7	1.9
7	0	319	12.5	12.5	7.5	8.8	2.6

* The males remained in the breeding cages for 3 days in Level 1, 2 days in Levels 2 and 3, and 1 day in Levels 4, 5, 6, and 7.

to 10,000,000 Strontium Units (S.U), or 10,000 times the occupational Maximum Permissible Concentration (M.P.C), and the lowest is 10,000 S. U., or 10 times the occupational M.P.C The present procedure involves mixing the ground meal with gelatin to facilitate shaping and hardening, sodium propionate to prevent the growth of mold, a pure food dye to provide color identification, water to give the desired consistency, and Sr^{90}. The wet mash is pushed through a converted meat grinder, which extrudes the food through five tubes. Pellet-size pieces are cut and then dried at 60 to 70°C for 48 hr. Control rations are prepared in similar fashion The evenness of mixing of the Sr^{90} has been tested by ashing and counting the disintegrations from a number of pellets from several batches The coefficient of variation was 2.4%. It is anticipated that a laboratory pelleting machine will soon replace this rather laborious food manufacturing process.

Animal production and maintenance Adult CF No. 1 female mice were housed 10 per stainless steel cage and given control food uncontaminated with Sr^{90}. One male mouse was introduced into each cage and was permitted to remain there for 24 hr Nineteen days later the pregnant females were isolated in confinement cages (stainless steel boxes containing sterile wood shavings), and the remaining mice, plus additions from stock to return the number to 10 per cage, were provided with Level 6 food (see Table 7). Two days later the male mice were returned to the breeding cages, and they were removed 24 hr. later. The males received food containing Sr^{90} only during their stay in the breeding cages

Nineteen days after this second breeding period the pregnant females were isolated in confinement cages, where they continued to receive Level 6 food. The remaining mice, plus some additions from stock, were changed to Level 5 food, and two days later the males were reintroduced to the cages. This routine was repeated, at increasing levels of Sr^{90}, until the series of dose levels was complete

The male mice and those females that had not produced litters were discarded. Mice with nonviable litters were maintained as post-breeding females at the Sr^{90} level they had reached when their litters were conceived. Mice with viable litters were added to this group when their young were 35 days old, or earlier if their litters were gone before that time.

The newborn mice were weighed as a group, and some males were selected for Sr^{90} and Ca analyses At 7-day intervals the bedding material was replaced, the young were again weighed, and additional males were taken for analysis. At 35 days the young mice were ear-marked, weighed individually, sexed, housed in stainless steel cages, and were provided with food at the appropriate Sr^{90} level The male mice remaining after 35 days have been removed periodically for analysis and autoradiographic studies. Males are being used exclusively for this purpose because they are notorious fighters and, consequently, useless in long-term toxicity experiments.

The experimental routines and maintenance procedures for the post-breeding and Sr^{90} -reared females are the same as those employed in our previous studies of the effects of internal emitters in mice. The animals are observed daily and weighed and examined individually at 1-month intervals. Moribund animals are killed with sodium pentobarbital after a peripheral blood sample has been taken, and autopsy is followed by skeletal roentgenography. Certain tissues are always taken for histological study, and others are taken as indicated.

Results

Considerations of space and man-power made it impossible to breed all of the groups at the same time. The procedure used was selected as the one that would make the best use of the available adult females while still producing all the young in a given group at the same time. The major disadvantage of the method is that comparisons of reproductive capacity among groups are not entirely valid. One reason is that at each successive breeding a large part of the population consisted of animals that had not bred when previously exposed. However, since the "average" mouse will mate only one day out of five, and since 21 days elapsed between breeding trials, it would be expected that animals in estrus at any period would not have been receptive at a previous breeding period. Another reason for caution in comparing the groups for reproductive capacity is that Level 1 mice were bred four months after the control, or Level 7, mice. Not only is four months a significant portion of the reproductive life of a mouse, but seasonal variation in breeding has often been noted in this species. In addition, manifestations of disease within a colony often fluctuate in severity from month to month.

The data in Table 7 should be examined with the above reservations in mind. The variations in the percentage of females producing litters and the percentage of litters with no live young are probably due to an endemic intestinal infection, which became apparent when the first litters were born. A few lactating mice became ill and failed to care for their young. A short time later some cages of breeding females developed the typical symptoms, primarily pallor, lethargy, and diarrhea. These animals did not become pregnant. The litters that were produced were large, on the average, and their size did not seem to be influenced by the level of Sr^{90} in the diet or by the age of the parents. The number of females reared to 35 days of age also did not vary substantially among the groups. However, the average number of females per litter reaching 35 days of age was only 2.4, whereas 8.1 young, or a calculated 4 females, were born. This loss of an average of 1.6 females per litter was due primarily to the infection mentioned above.

The numbers of post-breeding females and Sr^{90} -reared females that comprise the long-term toxicity study are listed in Table 8. Most of the

TABLE 8

Mortality and reticular tumors at 200 days

Level	$\frac{\mu c\ Sr^{90}}{g\ Ca}$	Post-breeding females			Sr^{90}-reared females		
		Number	Mortality, %	Reticular tumors, %	Number	Mortality, %	Reticular tumors, %
1	10.0	39	15.4	0	94	14.9	1.0
2	5.0	21	4.8	0	53	2.7	0
3	2.5	46	4.4	0	128	9.4	0.8
4	1.0	20	15.0	5.0	48	2.1	2.1
5	0.1	38	5.3	0	92	4.4	2.2
6	0.01	31	3.2	0	70	4.3	1.4
7	0	31	3.2	0	105	2.9	1.0

deaths that occurred among the dams during the first 200 days could be attributed to the intestinal infection. At autopsy the distended caecum was found to contain yellowish fluid, and the very pale, thick-walled colon typically contained only mucus. The livers were essentially normal. One post-breeding mouse had a tumor of the blood-forming tissues. No other neoplasms were found.

Most of the deaths between 35 and 200 days of age among the Sr^{90}-reared females were associated with the same gastrointestinal symptoms. Tumors of the reticular tissues appeared more frequently than among the dams during this interval, but no other neoplasms were seen. No skeletal abnormalities have been detected to date, but there is an impression that the Level 1 mice may be somewhat smaller than the others. However, no bone measurements have been made as yet, and the weight data have not been examined. Another important phase of the experiment, which will be reported when the results are available, concerns the analytical and autoradiographic study of the males.

TRITIUM LABELING OF ORGANIC COMPOUNDS OF BIOLOGICAL INTEREST

C. F. Decker, William P. Norris, and Walter E. Kisieleski

Labeling of organic compounds by the self-irradiation method described by Wilzbach et al.[1] is being investigated. One of these, l-proline, has been labeled. When l-hydroxy proline was subjected to the same conditions of irradiation it was found not to be labeled.

Experimental and Results

One gram of commercial l-proline or l-hydroxyproline was exposed to approximately 3 c of gaseous tritium for 3 days. Immediately after irradiation, the compounds were examined by paper chromatography on Whatman No. 1 filter paper. Two different solvent systems were employed – butanol:water:acetic acid and 77% ethanol. Ninhydrin-positive spots were identified using a modified reagent.[2] Chromatography of the irradiated l-proline in either solvent system yielded only one ninhydrin-positive spot which had radioactivity associated with it. However, with l-hydroxyproline, no radioactivity was found associated with the ninhydrin-positive area. These spots were identified as l-proline or l-OH proline by co-chromatography of an authentic sample of either imino acid. The strip containing the l-proline showed a second faint yellow spot (unidentified) which was not labeled. Considerable radioactivity was also found to be associated with the origin and solvent front.

The tritiated prolines were purified either by paper chromatography followed by elution with water or on a Dowex 50 (hydrogen form) column using 1 N HCl as the eluting agent. The resulting preparations in either case were examined by paper chromatography and found to be pure. With l-proline there was no radioactivity either at the origin or at the solvent front.

The results are shown in Figure 5. The presence of considerable quantities of radioactive impurities in A can be seen from the trailing of the photographic darkening from the origin to the solvent front. A single spot which corresponds to l-proline is apparent in B.

Radiochemical assay of the tritiated l-proline indicated that the specific activity of the crude material was approximately 40 μ c/mg. The specific activity of the purified material was 1.5 to 2 μc/mg.

Figure 5. Radioautograph made from paper chromatograms containing A, l-proline immediately after tritiation, and B, the same material after purification on Dowex 50.

References

1. Wilzbach, K. Tritium-labeling by exposure of organic compounds to tritium gas. J. Am. Chem. Soc. 79 1013 (1957).

2. Kolar, M. G. and H. R. Roberts. A new reagent for the detection of hydroxyproline on paper chromatograms. Arch. Biochem. Biophys. 70 620-622 (1957).

DELAYED EFFECTS OF X-IRRADIATION IN CHICKENS

S. Phyllis Stearner and Emily J. Christian

Acute radiation mortality in young chicks can be modified by various prophylactic techniques, some of which have also been tested and proved effective in mammals. We have reported the effectiveness of partial body shielding, low oxygen concentration, and epinephrine in reducing initial mortality (0 to 2 days) as well as deaths occurring after 2 days postirradiation in chicks.[1,2] During the first half of 1953 various combinations of these treatments were tested for protective action. Mortality in the acute period was followed and, in addition, some of the 30-day survivors were kept for observation of delayed changes. At this time about one-third of the original group of 30-day survivors is still alive. This discussion is, therefore, a preliminary summary of observations over a period of 2000 days after irradiation.

Male white leghorn chicks, at 3 to 4 days after hatching, were exposed to X-rays at a dose of 1200 r, delivered in 35 min. Irradiation conditions and protective procedures have been previously described.[1,2] Three types of protective treatments were used. Partial body shielding involving about 10% of the body protected the ventral abdominal region (primarily liver) or the dorsal lumbar region including kidneys, adrenals and part of the spine. An atmosphere of 7.5% oxygen was used to produce hypoxia during irradiation. Protection was also provided by administration of epinephrine in peanut oil immediately before and after exposure. These treatments were used singly and in combination to produce the best possible radiation protection in the acute (30-day) period.

Mortality in the initial (0 to 2 days) and the later (3 to 30 days) acute periods in the protected irradiated groups is shown in Table 9. Mortality during these periods after single total-body X-ray exposures delivered in 35 min (Figure 6) indicates that less than 1% survival is to be expected at 30 days after 1200 r given in 35 min. Thus, significant survival at this dose level in irradiated protected groups may then be considered to indicate a significant degree of protection. Combination of the three procedures provided greatest protection, but in no group was the effective dose reduced by as much as 50%.

At 30 days, birds to be observed for delayed effects were numbered and weighed. Since that time, the animals have been caged individually and maintained under conditions standard for the animal quarters. Weights were taken approximately monthly and general condition noted at intervals. Decedents were autopsied, gross changes recorded and, in most cases, tissues taken for histopathologic examination.

TABLE 9

Effects of protective techniques on acute mortality after 1200 r X-irradiation (35 r/min)

Shielding	Additional protective treatment*	Number	Mortality, %		Survival at 31 days %
			0-2 days	3-30 days**	
None	None	67	94.0	100.0	0
	7.5% O_2	47	74.5	75.00	6.4
	Epinephrine	42	59.5	58.8	16.7
	7.5% O_2, epinephrine	45	53.3	42.9	26.7
Kidney region	None	42	69.0	53.8	14.3
	7.5% O_2	38	31.6	46.2	36.8
	Epinephrine	46	19.6	40.5	47.8
	7.5% O_2,epinephrine	39	15.4	27.3	61.5
Abdomen	None	32	78.1	100.0	0
	7.5% O_2	32	56.3	50.0	21.9
	Epinephrine	31	51.6	86.7	6.5
	7.5% O_2, epinephrine	31	25.8	69.6	22.6

* O_2, 7.5% during irradiation; epinephrine, 5 mg/kg in peanut oil intramuscularly immediately before and after irradiation.

** Based on number surviving at 3 days.

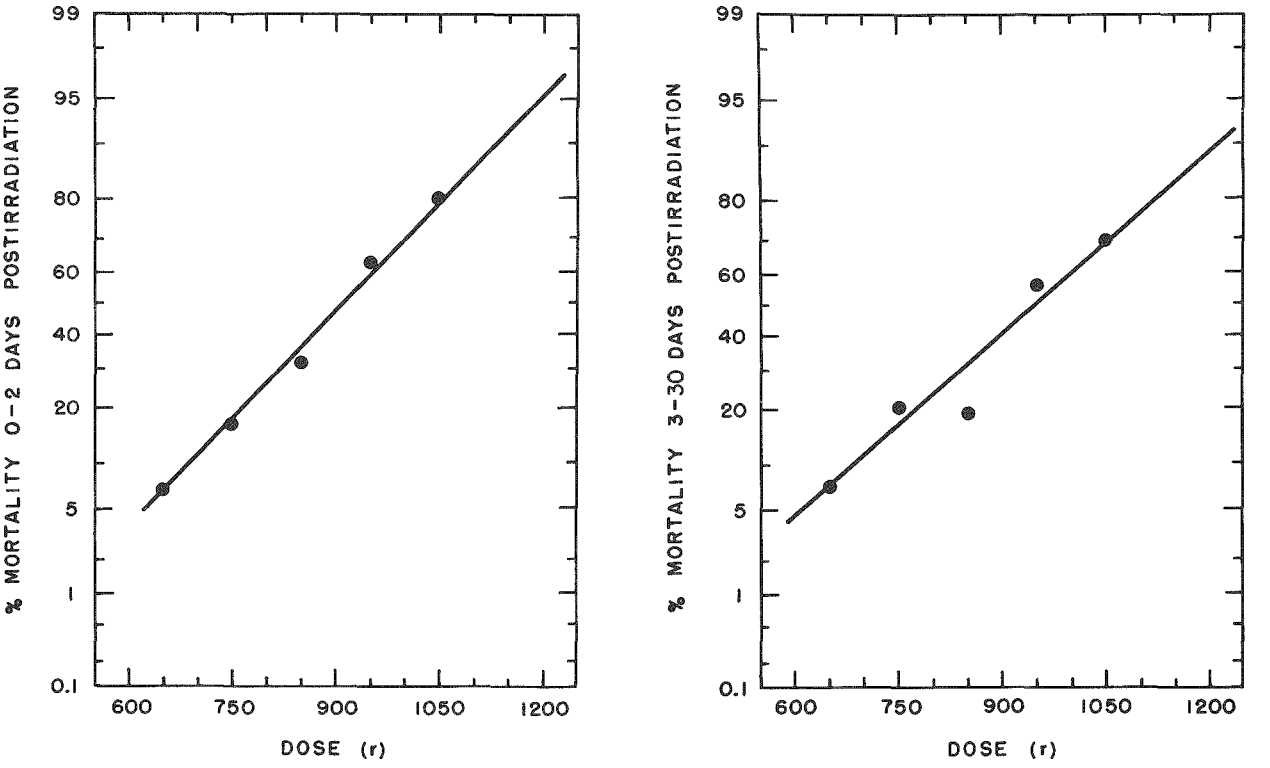


Figure 6. Dose mortality curves following X-irradiation of 3-day chicks.
A. Initial (0-2 day) mortality; B. Later (3-30 day) mortality.

Based on time distribution of deaths and associated pathology, three chronic periods are indicated at this time in this group of birds: 3 to 11 months (no deaths between 30 and 85 days), 1.0 to 4.5 years, and more than 4.5 years. Mortality in these periods and survivors at 2000 days are shown in Table 10. Deaths experienced during the first year are of special interest. Most birds that died between 3 and 11 months showed severely atrophied livers with large accumulations of ascitic fluid. It may be noted (Table 10) that only 1 death occurred among those in which the abdomen was shielded during irradiation. During the following 3.5 years there were relatively few deaths but again only one was from a group in which the abdomen was shielded, but most recent mortality has included several deaths in these groups.

TABLE 10

Effects of protective techniques on delayed mortality after 1200 r X-irradiation (35 r/min)

Shielding	Additional protective treatment*	Number observed after 30 days	Mortality			Survivors at 2000 days
			3-11 months	1.0-4.5 years	4.5-5.5 years	
None	7.5% O_2	None				
	Epinephrine	4	1	2		1
	7.5% O_2, epinephrine	6	3	2	1	0
Kidney region						
	None	5	5	-		0
	7.5% O_2	6	3	2		1
	Epinephrine	9	5	1	1	2
	7.5% O_2, epinephrine	6	2			4
Abdomen	None	No survivors				
	7.5% O_2	9	1		3	5
	Epinephrine	2		1		1
	7.5% O_2, epinephrine	5			2	3
Total		52	20	8	7	17
Controls		6		1**	2	3

* O_2, 7.5% during irradiation; epinephrine, 5 mg/kg in peanut oil intramuscularly immediately before and after irradiation.

** Accidental fracture.

The principal protective effect of kidney shielding appears to be on acute mortality, specifically the initial response, resulting in greater survival at 30 days (Table 9). During 3 to 11 months postirradiation, however, mortality in these groups was high (more than 50%), and comparison with total body irradiated groups indicated no protection from kidney shielding.

Abdomen shielding alone resulted in little reduction in acute mortality, but when combined with other treatments a sufficient number survived the acute period to permit study of later effects. The mortality between 3 and 11 months postirradiation may have some relation to the liver pathology observed during this period and to direct exposure of the entire liver. The low mortality in groups with the abdomen shielded suggests that such liver damage may be at least partly a direct radiation effect which is modified by shielding.

Weight curves of the control and two irradiated groups are shown in Figure 7. Two phases are suggested from the control curve: An initial period of rapid growth for about 200-250 days, and a later slow weight increase which has continued for the duration of observations. The mean weights of the various irradiated groups that survived more than a year show only minor differences. Apparently, irradiation did not alter the duration of the period of rapid growth but reduced the growth rate; thus the maximum weight attained at the end of the rapid growth phase was far

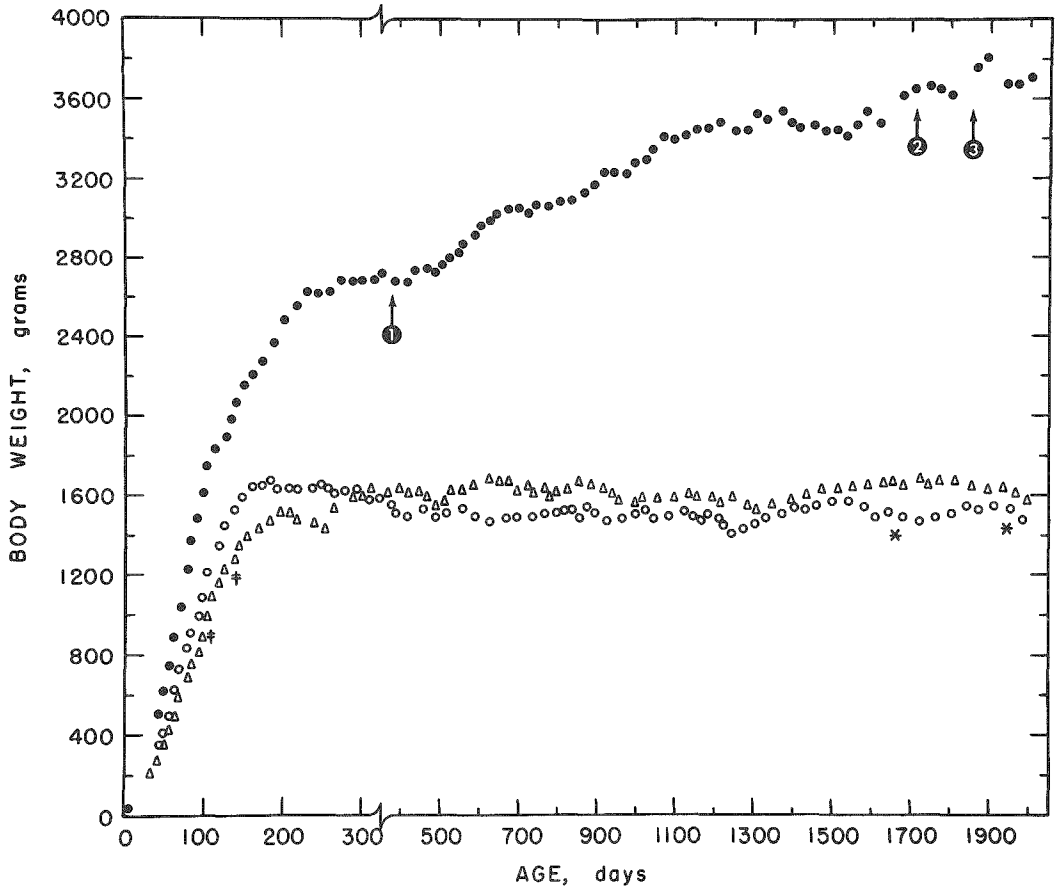


Figure 7. Growth of control and X-irradiated chicks, exposed at 3 days after hatching. ●, control (1, accidental fracture, 2 and 3, death); △, kidney shielding, 7.5% O_2, 5 mg/kg epinephrine in peanut oil before and after irradiation (‡, death); ○, abdomen shielding, 7.5% O_2, 5 mg/kg epinephrine in peanut oil before and after irradiation (*, death).

below the control level. At this time the length of the long bones in irradiated birds was much less than that of controls. Abdomen shielding, which reduced mortality during this period, did not modify the growth rate. After the period of rapid weight increase (about 250 days), irradiated groups showed no further increase such as was seen in the controls. The nature of the processes involved in the later component of the growth curve and their relation to delayed radiation effects are of special interest.

All irradiated birds have shown some development of male sex characters, e.g. enlarged comb and wattles, and modified head and saddle feathering. Apparently there was development and persistence of interstitial cells in the testes although in all cases that have been examined seminiferous tubules contained no spermatogonia.

Lymphomatosis has not been identified among irradiated birds but was seen in one control that died at 1775 days. During 2000 days of observation, one teratoma has been recorded in a control and one sarcoma (identified grossly but not yet verified) in an irradiated animal.

References

1. Stearner, S. P., E. J. Christian and A. M. Brues. Modification of the radiation syndrome in the chick by partial body shielding Radiation Research 1 270-281 (1954).

2. Stearner, S. P., E. J. Christian and A. M. Brues. Protective action of low oxygen tension and epinephrine against X-ray mortality in the chick. Am. J. Physiol. 176 455-460 (1954).

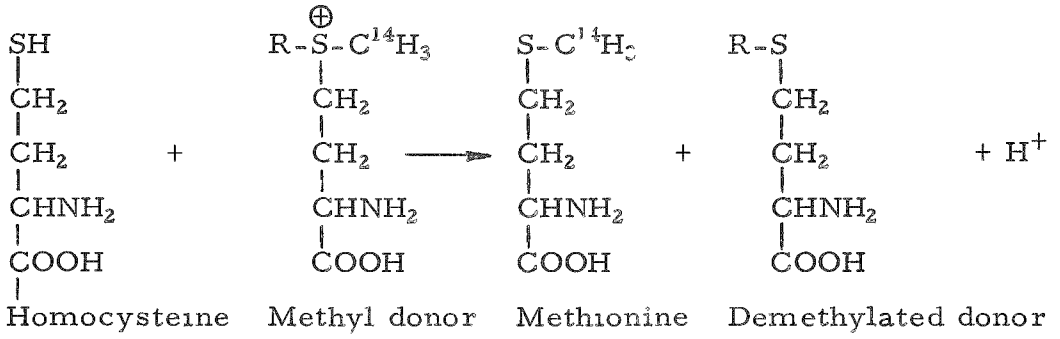
PROGRESS REPORT. THE HOMOCYSTEINE TRANSMETHYLASE SYSTEM

Stanley K. Shapiro and David A Yphantis

A new method for the assay of enzymatic activity of various homocysteine transmethylases has been described previously (1,2) Details of this method and its applications are presented in this report

Results and Discussion

The assay was designed to measure the following reaction of methyl methionine or S-adenosyl methionine, R represents $-CH_3$ or adenosine.



The method depends only on an adequate procedure to separate the methyl donor and the methyl product This is accomplished by means of Dowex 50 columns in the Li^+ form: 99% of the radioactivity in 10 μM of S-adenosyl-L-methionine is retained on such columns, whereas 99.98% of the radioactivity in 10 μM of S-methyl-L-methionine is retained on the columns after washing with 5 ml of distilled water. Conversely, radioactive methionine may be recovered quantitatively by washing the column with three 0.25-ml portions of distilled water. Thus enzymatic activity may be measured by placing aliquots of reaction mixtures on Dowex columns and measuring the radioactivity contained in the column effluents. Since methionine is the only radioactive product formed in the reactions with yeast extracts, the amount of radioactivity in the effluent is directly proportional to the methionine produced Reaction mixtures without L-homocysteine or without enzyme are used to obtain blank values.

The amount of radioactivity may be measured by any of the conventional procedures, such as plating aliquots of deproteinized solutions. Liquid scintillation counting has proven most convenient because the entire solution may be used without any pretreatment. This minimizes errors due to sampling and plating. The absolute efficiency of counting methionine-$C^{14}H_3$ in our system is about 40% and is unaffected by the presence of crude yeast extract or reasonable levels of unlabeled substrates

Figure 8 shows the time course of the transmethylation of L-homocysteine with S-methyl-L-methionine as the methyl donor using baker's yeast as the source of enzyme. At each of the three enzyme levels used the curves obtained are linear throughout the time of incubation.

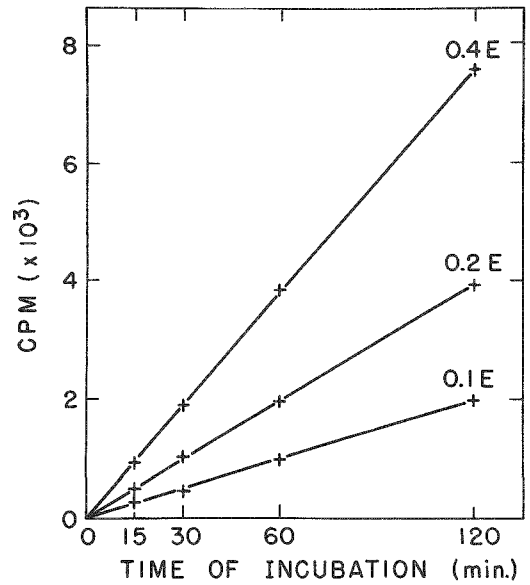


Figure 8

S-Methylmethionine-homocysteine transmethylase activity as a function of time. Reaction mixtures contained per ml: 8 μM L-homocysteine, 8 μM S-methyl-L-methionine ($C^{14}H_3$) (200,000 cpm/μM) and varying amounts of a 1:5 dilution of yeast enzyme. The mixtures were prepared in 0.1 M phosphate buffer pH 6.4 and incubated at 35° C; 0.2-ml samples were removed at the designated time intervals and the radioactivity of the methionine was determined as described. (Specific activity of methionine, 100,000 cpm/μM).

Figure 9 shows the data plotted as a function of enzyme concentration. At each of the four time intervals used, the curves are linear. Under usual assay conditions, this linear relationship is maintained only when the level of methionine produced is under 0.5 μM per ml of reaction mixture. Above this level there is a gradual inhibition of the reaction.

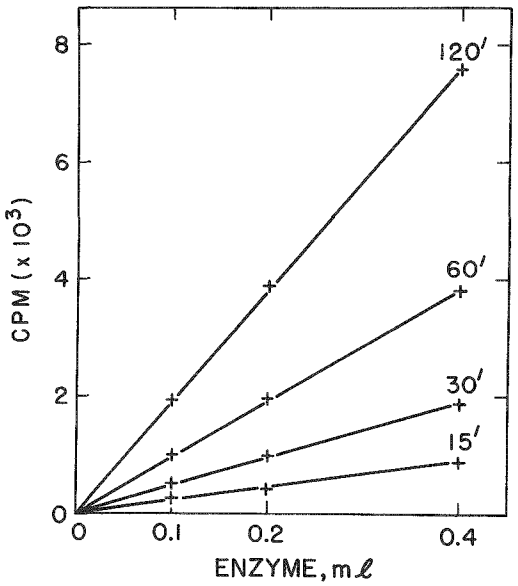


Figure 9

S-Methylmethionine-homocysteine transmethylase activity as a function of enzyme concentration. Conditions were as described for Figure 8.

With S-methyl-L-methionine as the methyl donor, the standard deviation of a routine determination of transmethylase activity has been about 3%. At the present time we are able to use S-methyl-L-methionine at such a specific activity (700,000 cpm per μmole) that 0.0004 μM of the resultant methionine (specific activity of 350,000 cpm per μmole) may be detected at a level of 2 times background in a 0.2-ml sample. Thus we are able to work in the range of 0.002 to 0.5 μM of methionine formation per ml of reaction mixture. There seems to be no reason why this sensitivity could not be greatly increased if necessary by simply preparing the methyl donor with a higher specific activity. Compared to the previous colorimetric methods this procedure for assaying transmethylase activity has improved sensitivity more than 100-fold and has greatly simplified the manipulations required for assay.

Figure 10 demonstrates the application of the tracer assay in a determination of enzyme activity as a function of pH. The optimum range of activity is between pH 6.2 and pH 7.0.

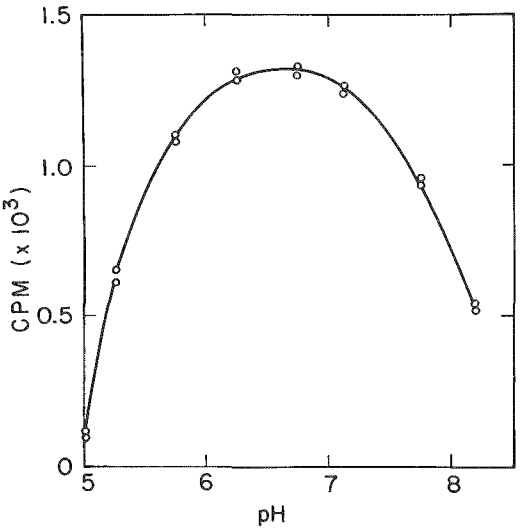


Figure 10

S-Methylmethionine-homocysteine transmethylase activity as a function of pH. Conditions were as described for Figure 8 except that the buffer was varied to attain various pH levels. All tubes contained 0.1 ml of a 1:5 dilution of yeast enzyme per ml reaction mixture and the incubation time was 90 min.

The tracer assay was used to conduct a survey of various cells for enzyme activity. The positive results are shown in Table 11. A number of strains of Aerobacter aerogenes were investigated, and all yielded cell-free extracts of high activity. Although extracts of strain K-12 of Escherichia coli were quite active, we have been unable to demonstrate activity in extracts prepared from strains B and W. Rat kidney preparations had low activity, but those of rat liver were comparable to the yeast preparations. Under identical conditions acetothetin was four times as active as S-methyl-L-methionine as a methyl donor. No enzyme activity has been detected in cell-free preparations of rat brain or heart. Activity was not demonstrable with cell-free extracts of cabbage, peas or lily of the valley.

TABLE II

A comparison of the activity of various homocysteine transmethylases*

Source of enzyme	Methyl donor		
	Acetothetin**	S-Methyl-L-methionine-$C^{14}H_3$	S-Adenosyl-L-methionine-$C^{14}H_3$
Rat kidney	< 0.2	0 05	< 0 002
Rat liver	2 0	0 5	0 1
S cerevisiae	< 0.2	0 8	0 2
E. coli K 12	< 0.2	0 7	0 15
A aerogenes	< 0 2	1 0	0 3

*All quantities are expressed as μmoles of methionine per mg protein per hour incubation Each reaction mixture contained 8 μM L-homocysteine and 8 μM methyl donor per ml. Reaction mixtures were made up in 0 1 M PO_4 buffer, pH 6 4, and incubated at 35°. S-Methyl-L-methionine ($C^{14}H_3$) was used at a specific activity of 400,000 cpm/μM, methionine was determined on the basis of a specific activity of 200,000 cpm/μM S-Adenosyl-L-methionine ($C^{14}H_3$) was used at a specific activity of 425,000 cpm/μM, the resultant methionine in this case has the same specific activity as the methyl donor.

**The methionine produced with acetothetin as the methyl donor was determined by the nitroprusside test.

Table II also shows the results obtained with the new method using S-adenosyl-L-methionine as the methyl donor for the transmethylation of L-homocysteine by various cell-free extracts. With S-methyl-L-methionine, one would expect 2 moles of methionine per mole of methyl donor, whereas with S-adenosyl-L-methionine the theoretical yield would be 1 mole methionine per mole of methyl donor. However, the apparent activity of S-methyl-L-methionine exceeds the activity of S-adenosyl-L-methionine by a factor of 3 to 5 Thus S-methyl-L-methionine appears to be the better methyl donor for the transmethylation of L-homocysteine under the described conditions. The amount of methionine formed by liver extracts in the transmethylation of L-homocysteine with S-adenosyl-L-methionine is easily detected by the tracer assay although this possibly important role of S-adenosyl-L-methionine would be difficult to detect with colorimetric procedures.

The sensitivity of the assay makes it possible to detect very small amounts of enzyme activity However, its greatest advantage in our work is that it is possible to assay many enzyme preparations quickly and accurately. Although it is possible to use extremely short incubation times in

our routine assays, we have found it more convenient to adjust the specific activity of the methyl donor so that incubation of reaction mixtures for 30 min yields an accurately measurable amount of radioactivity in the resultant methionine. It is obvious that this method could be applied advantageously with only minor modifications to studies involving any of the known homocysteine transmethylases

In principle such a method for enzyme assay could be used in many other reactions where present assay methods are unsatisfactory. The only prerequisite is a method that gives a quantitative separation of a substrate and a reaction product, permitting radioactivity to be used as a measure of enzymatic activity.

References

1. Shapiro, S. K., and D. A. Yphantis. Progress report: The homocysteine transmethylase system. Biological and Medical Research Division Semiannual Report. Argonne National Laboratory, ANL-5841 (1957). pp. 62-63

2. Shapiro, S. K., and D. A. Yphantis. Assay of S-methylmethionine and S-adenosylmethionine homocysteine transmethylases. Biochim. Biophys. Acta 36 241-244 (1959).

A PUNCH-CARD SYSTEM FOR THE STUDY OF ELECTRON MICROGRAPHS

Miguel Mota*

It is frequently necessary in electron microscope work to study a large number of photographs. If the problem involves different variables such as irradiation of the material, time of recovery, different fixatives or details of technique, and several structures, the process of sorting for comparative study all pictures showing results of a given treatment or structure is a tedious and time-consuming one. A punch card system developed in connection with our own work has proved successful and can easily be applied to any similar problem.

Since it is convenient to locate immediately the print of any given negative we now make two prints from each negative in the standard 8 x 10 inch paper. One is filed in order by number, which permits immediate location of any number. The other is used for the punch card system.

Of the simple desk punches available we use one that makes a total of 7 holes in a length of 26 cm. By the use of the 8-inch edge of the print (where the square negative leaves a wider margin), 5 holes can be punched. By flipping over the print and punching it again we obtain 5 more holes. As in any punch card system it is convenient to cut one corner of the print to ensure quickly that they are stacked in the right position.

If not more than 10 variables are involved, the simplest system is to code them from A to J and assign a hole to each letter. Sorting once will provide all of the prints, and only those, of the desired variable.

If more than 10 variables are involved, it is necessary to cut more than one hole for each variable in each print. With two holes and the different combinations of the 10 letters, a total of 45 variables can be studied, a number usually enough to cover the needs of any experiment. If more variables are involved one can either use combinations of three letters or punch the other sides of the photograph. Even with the large holes as made by the punch used, this does not take more than 15 mm of the margin.

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SPECIFIC CHROMOSOMAL CONTROL OF THE MASS OF THE NUCLEOLUS AND OF THE CYTOPLASM

A. Longwell* and G Svihla

In a species in which it is possible to control the nature and extent of aneuploidy, it is feasible to determine whether the protein and ribonucleic acid content of the developing nucleolus is under direct chromosomal control. If so, it should then be possible to determine whether this control is a general function of all the chromosomes or of specific chromosomes to a greater extent than others. If nucleolar mass can be altered by changing the chromosomal constitution of cells, then information might be obtained concerning the proposed direct nuclear control - or more specifically the nucleolar control - over cytoplasmic protein synthesis, as well as the extent of this control should it exist.

Hexaploid wheat (Triticum vulgare) because of its allopolyploid nature, can tolerate a considerable degree of aneuploidy. Sears(1) has obtained a complete aneuploid series in this plant including monosomes, trisomics, tetrasomics and nullisomics for all twenty-one chromosomes of this species. (Monosomic: having the chromosome present in a single dose rather than the normal two doses; trisomic: having the chromosome present in three doses; tetrasomic: having the chromosome present in four doses; nullisomic: having the chromosome altogether lacking.) With the Cooke-Dyson interference microscope determinations have been made of the dry mass of the nucleolus and of the cytoplasm of plants with altered numbers of nucleolar chromosomes and plants with altered numbers of non-nucleolar chromosomes Inasmuch as the dry mass is indicative in these cases of the protein content of the cell, and since protein is formed under the influence of RNA, such a study can be interpreted in terms of the RNA and protein synthetic mechanisms of the cells

The microsporocytes of plants tetrasomic for two different non-nucleolar chromosomes did not differ in nucleolar or cytoplasmic dry mass from that of normal cells. Plants tetrasomic for two of the stronger nucleolar chromosomes, however, showed a considerable, statistically significant increase in the mass of both nucleoli and cytoplasm. When a plant tetrasomic for a nucleolar chromosome is missing a pair of non-nucleolar chromosomes, the total chromosome number then equaling that of a normal plant, the nucleolar and cytoplasmic mass fails to differ from that of the regular nucleolar chromosome tetrasomics. The cells of plants missing a pair of one of the strongest nucleolar chromosomes did not differ from normal in the aspects studied here, presumably because of the compensating

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*Resident Research Associate

effects of the two weaker pairs of nucleolar chromosomes, which in normal material are mostly latent. The average increases in mass in the cells of the plants tetrasomic for the two stronger nucleolar chromosomes are on the order of 100% in the case of the nucleoli and 50% in the case of the cytoplasm. All of these findings agree with independent measurements of nucleolar volumes and counts of newly-formed nucleoli before nucleolar fusion occurs.

These results indicate the control of nucleolar synthesis by specific chromosome types, these chromosomes being those which have been referred to in classical cytology as nucleolar chromosomes.(2) These chromosomes, or the nucleolar material which they synthesize, then exert an influence on the protein content of the cytoplasm out of all proportion to what might be expected on the basis that the extra pair of nucleolar chromosomes constitutes only $\frac{1}{22}$ of the total number of chromosomes present in the nucleus.

References

1. Sears, E. R. The aneuploids of common wheat. Univ. of Mo. Agr. Res. Bull. 572 (1954).

2. Crosby, A. R. Nucleolar activity of lagging chromosomes in wheat. Am. J. Bot. 44 813-822 (1957).

PROGRESS REPORT: DEUTERIUM OXIDE INTOXICATION IN RATS

I. Effect of D_2O on Peripheral Blood Cells

John F. Thomson and Florence J. Klipfel

In earlier experiments with D_2O-treated rats, we had often observed what seemed to be a severe anemia, as evidenced by the decreased volume of packed erythrocytes. We thought that a systematic study of the changes in numbers of peripheral blood cells would be profitable.

Sprague-Dawley female rats, 4 to 6 months old, were used in these experiments. They were given 50% D_2O ad libitum until one or more rats in the group died from the effects of deuteration (24 to 30 days). Blood counts were made at weekly intervals. Since the tails of D_2O-treated rats become necrotic, it was necessary to obtain blood by heart puncture. Urine was collected, also at weekly intervals, and assayed for deuterium.*

No significant changes were observed in rats until the D_2O concentration in the body exceeded 20%. As can be seen from Figure 11, the erythrocyte count then began to fall to about 50%, while the reticulocyte count increased more than two-fold. In 2 of the 6 rats in this group, the red cell count fell below 2,000,000/mm^3; 1 of these rats died, but the other recovered when given H_2O instead of D_2O to drink. After restoration of H_2O to the animals, recovery was apparently complete within 4 to 6 weeks.

The behavior of the white blood cells was considerably less consistent. In most cases, there was first a slow, then a sudden drop in lymphocytes at 25% replacement of the body fluids by heavy water, with a significant increase in granulocytes, so that the total white count did not change greatly in the course of deuteration. There were in some rats appreciable numbers of immature and abnormal white cells, but these findings were not consistently observed.

These observations differ from those reported by Gustavson and Häggqvist in mice.(1) These authors observed a 60% decrease in total white count, but only a 15% decrease in numbers of erythrocytes. However, their mice were drinking 50% D_2O for only 14 to 16 days, and with further ingestion a more marked anemia might have developed.

Although D_2O can thus suppress erythropoiesis, it is doubtful whether anemia is the cause of death in the majority of the animals; at least there is no apparent correlation between the degree of anemia and time of death.

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*Dr. Henry Crespi of the Chemistry Division carried out these analyses.

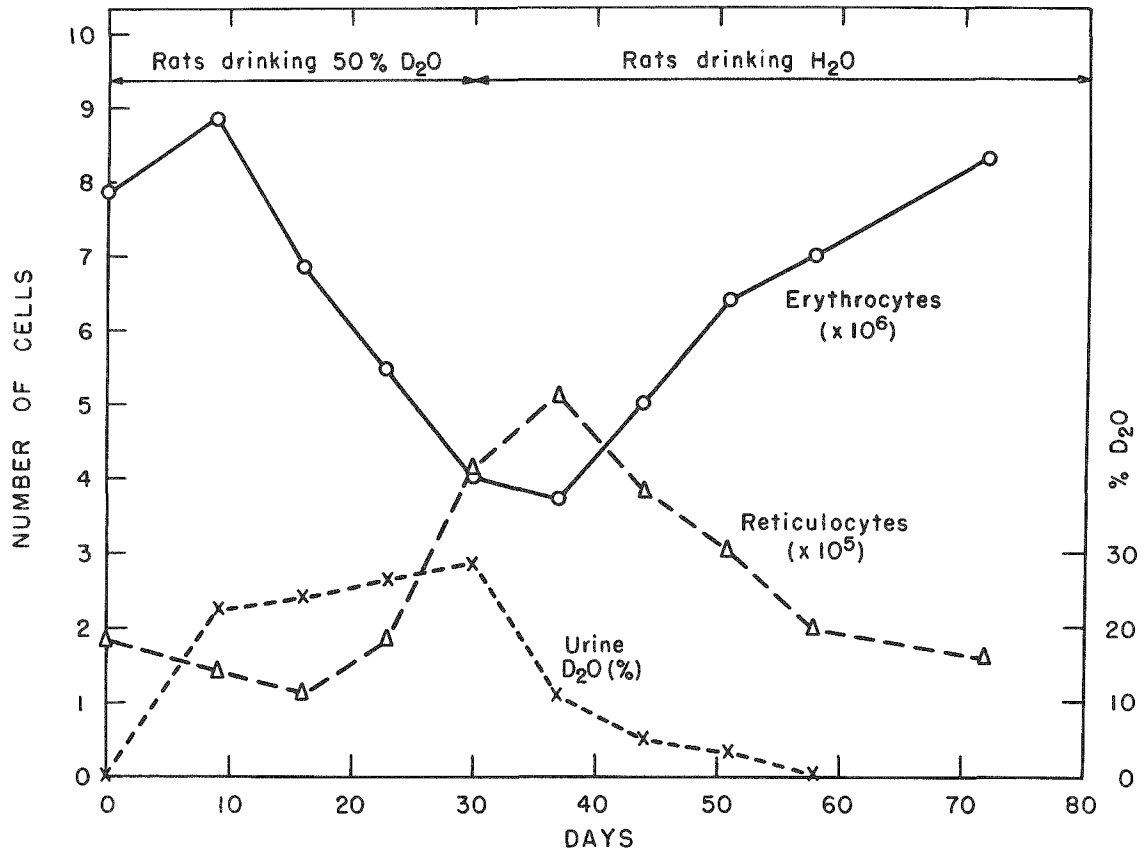

Figure 11. Changes in erythrocyte and reticulocyte count in rats drinking 50% heavy water. Averages of 6 rats.

Reference

1. Gustavson, K. H., and G. Häggqvist. Die Einwirkung von schwerem Wasser auf das Blutbild der Mause. Z. mikroskop. anat. Forsch. 63 388-97 (1957).

ELECTRON MICROSCOPE STUDIES OF SPERMATOGENESIS IN MOUSE AFTER X-IRRADIATION

I. Mitotic Spindle and Nuclear Structures in Sertoli Cells and "Cores."

Miguel Mota*

Although the mitotic spindle as seen under the electron microscope has been described by several authors,[1,2,3,4,5] complete knowledge on this cell structure is far from being attained. The present observations on the ultrastructure of the mitotic spindle and other nuclear structures, made in the course of a study of spermatogenesis in the mouse after X-irradiation, should therefore be of interest

Figure 12 shows the spindle in the primary spermatocytes of the mouse 24 hr after 1000 r of X-irradiation. These photographs cannot be considered good by the usual standards although it is not possible to say if

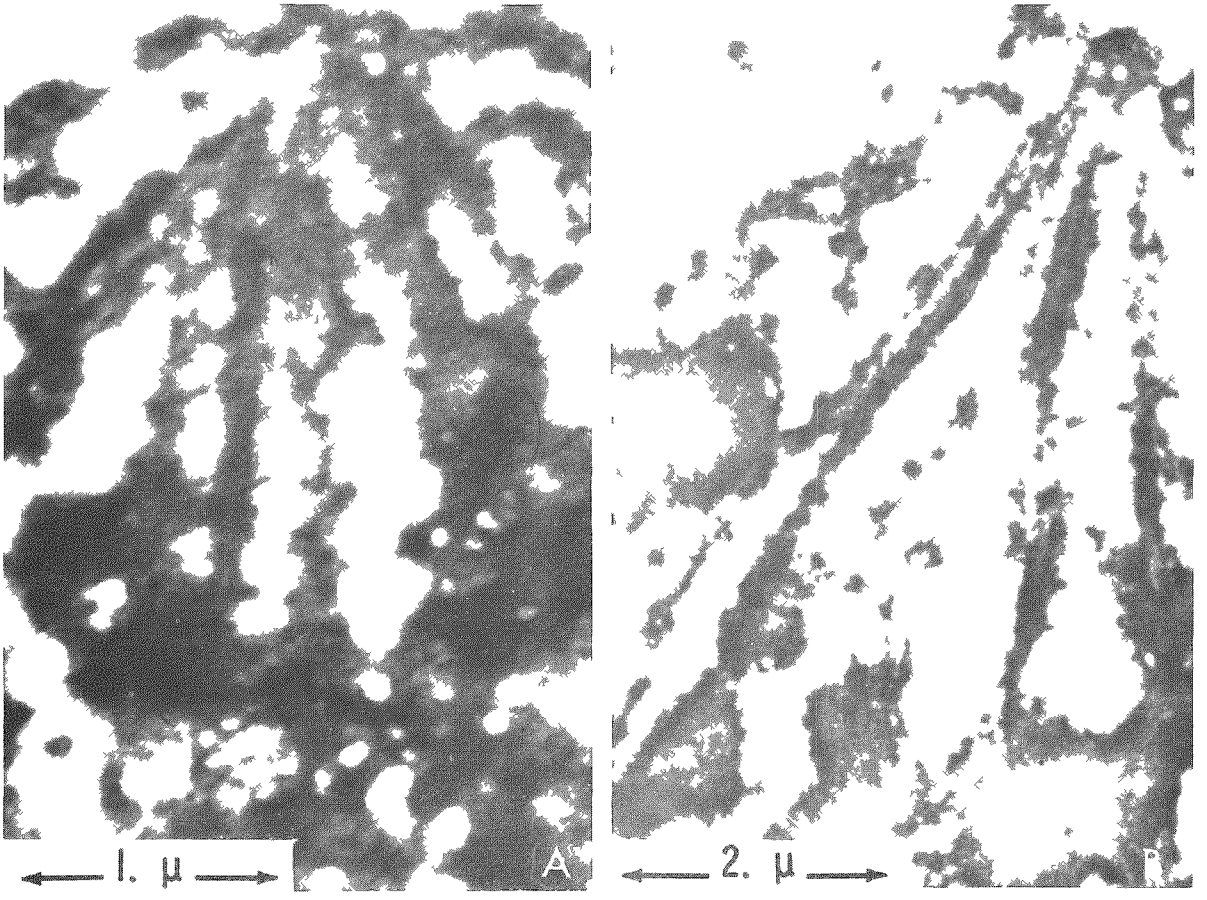

Figure 12. Two views of the achromatic spindle in primary spermatocytes 24 hr after 1000 r X-rays.

*Resident Research Associate.

the bad condition and tearing of the material results from fixation (doubtful), or as suggested in a personal communication of Prof. Yasuzumi, from dehydration (from too prolonged exposure to absolute alcohol), or from polymerization of the methacrylate. In these pictures, however, one can see that the spindle fibers show a peculiar resistance to whatever caused the destruction of the cytoplasmic arrangement. The spindle fibers appear to constitute an extremely tough organization or, at least, to be particularly resistant to the artifact abundantly shown in the pictures. This is in good agreement with the fact that the mitotic spindle can be isolated by disorganizing the cytoplasmic proteins by detergents.(2)

From these pictures and the above-cited papers the fact seems well established that the so-called chromosomal fiber is composed of two parallel bundles of fibrils. Each bundle presumably is attached to the centromere of a chromatid (half centromere) although no good evidence of this has been clearly presented. Each bundle is itself composed of double fibrils, leaving open again the idea that "spindle fibers" may be actually tubules.

The distance between the two parallel bundles of fibrils of each chromosomal fiber may be as high as about 2000 A. This is roughly the limit of resolution of the optical microscope, and that structure should then, theoretically, be visible with that instrument. It requires only adequate technique to be demonstrated because of the well known difficulty in observing the achromatic spindle. With the interference microscope, however, such a double structure is visible as described by Longwell and Mota.(6) This double structure was also visible although less clearly, in preparations of mouse testis fixed in Carnoy fluid, stained with Feulgen and Fast Green, and observed by phase-contrast microscopy in preparations kindly shown to us by Dr. B. R. Nebel.

A peculiar structure was observed in some resting stage cells consisting of a round region centrally located in the nucleus (Figure 13 A and B) and having in its periphery a ring of electron-dense material irregularly distributed. Attached to the outside of this region is the nucleolus, well-defined as the darkest structure in the cell and with vacuoles. The center of the round region appears rather clear with only a small number of slightly denser regions. The peripheral ring seems to be composed of partially despiralized chromosome material, and as these regions were first seen in material fixed a few days after irradiation, it was thought that they might be restitution nuclei. Restitution nuclei appearing some time after 1000 r acute localized X-irradiation have been described as the result of blockage of mitosis at metaphase I by Nebel and Dlesk(7) from optical microscope studies of mouse testis after irradiation.

Because of the well-known extreme lability of the achromatic spindle, its destruction or inactivation is to be expected with such a high dose as 1000 r of X-irradiation. Hence, the fate of a cell irradiated in metaphase or near that stage will probably be either death or restitution. The latter

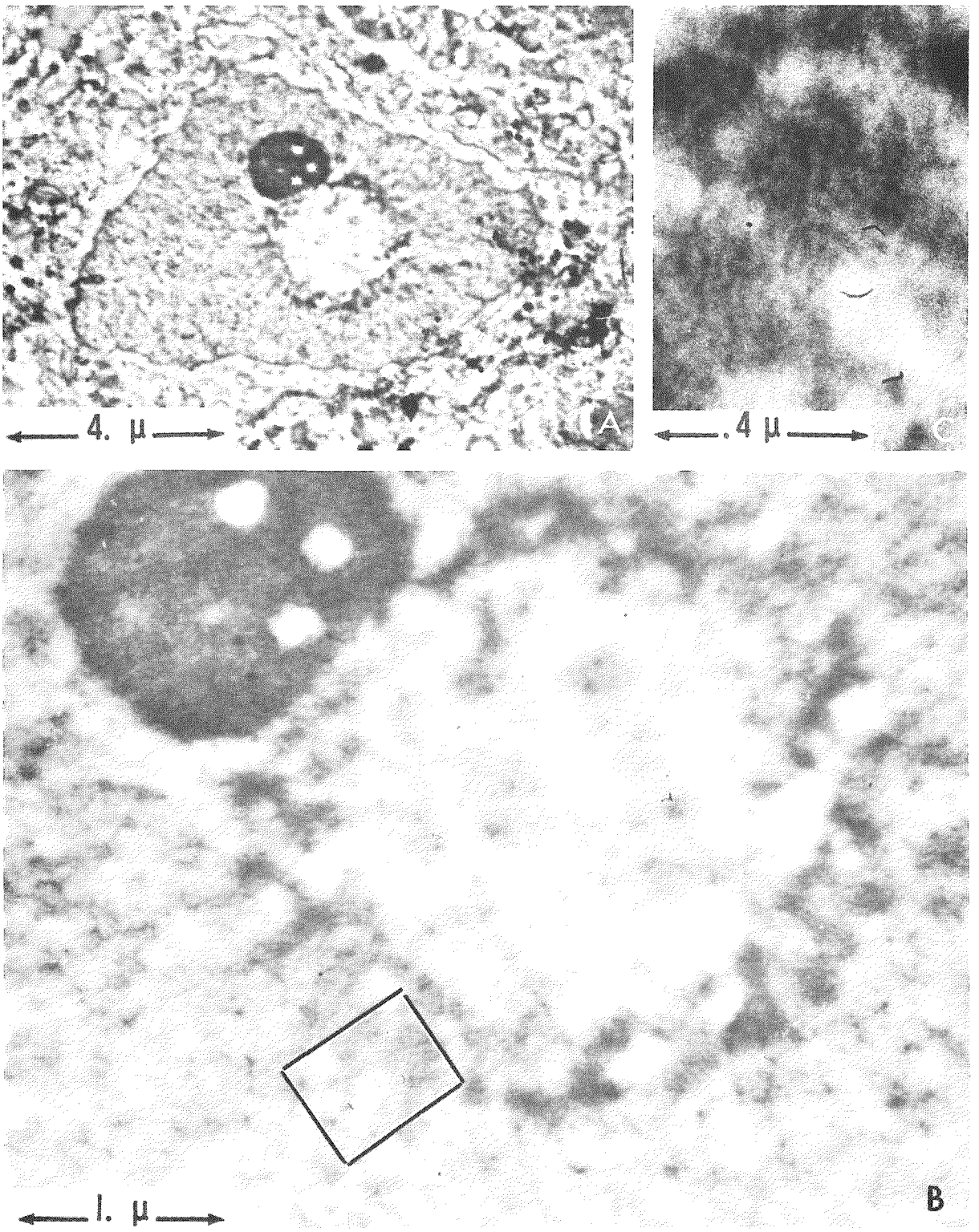

Figure 13. A, Nuclear structure in a Sertoli cell 7 days after 1000 r X-rays; B, part of A at higher magnification, showing the transition zone and the nucleolus; C, further magnification of the rectangle marked in B, showing the 200-A fibrils.

process resembles the telophase process in the despiralization of the chromosomes and the reappearance of the nucleolus: the chromosomes lose their individuality and the whole nucleus appears as a uniform mass. The process does not occur uniformly over the entire metaphase plate but is a centripetal one, as can be seen in motion pictures of living cells of plant endosperm by Molè-Bajer.[8]

The main difference in appearance between Bajer's restitution nuclei and those seen here is that here the central region looks almost clear, while in Bajer's pictures it is filled with only slightly despiralized chromosomes. The first cells of this kind to be studied were not clearly identified. Although they resemble Sertoli cells, it was thought that they might be restituted spermatocytes which, according to Nebel and Dlesk,[7] "can thoroughly mimick the appearance of Sertoli nuclei." These structures were later found also in control material in unmistakably identified Sertoli cells. The number of cases found makes it highly improbable that they are spontaneous restitution nuclei; therefore, this hypothesis has been almost entirely ruled out.

The density of the periphery of the round central region, which is higher than in other nuclear regions and lower than in the nucleolus, suggests that the material may be heterochromatin. The fact that it is always attached to the nucleolus, and that in the mouse the nucleolar chromosome is heterochromatic, also strongly suggests that this is really heterochromatic chromosome material in a stage of partial despiralization. However, we have no explanation for the perfectly round shape of this structure or for its clear central zone.

Either in restitution nuclei or in heterochromatic chromosome material, a partially despiralized structure is likely to be particularly suitable for the study of the arrangement of fibers inside the chromosome since the despiralization process is probably going on at a lower rate than in telophase. If so, such cells might provide help in solving what is, at present, the most pressing problem of morphology of chromosome structure. The observations made so far show the presence in this region of well-defined fibers about 200 A wide (Figure 13 B and C). They seem to correspond to the elementary 200-A fibrils that Ris[9] considers the basic morphological unit although more recently he has been able to resolve that unit in particularly favorable material and conditions.

In synaptic stages of primary spermatocytes, "cores" similar to those described by Moses[10] have been found repeatedly in control material. Our observations are consistent with those of other authors; the existence of such cores may be rather general.

References

1. Sedar, A. W., and D. F. Wilson. Electron microscopic studies on the normal and colchicinized mitotic figures of the onion root tip (Allium cepa). Biol. Bull. 100 107-115 (1951).

2. Mazia, D. Studies on cell division. In Cellular Biology, Fifteenth Annual Biology Colloquium, Oregon State College, 1954. Oregon State Chapter of Phi Kappa Phi, Corvallis, Oregon. pp. 3-14.

3. Porter, K. R. Changes in cell fine structure accompanying mitosis. In Fine Structure of Cells, Symposium, Leiden, 1955. New York Interscience Publ., Inc. pp. 236-249.

4. Amano, S. The structure of the centrioles and spindle body as observed under the electron and phase contrast microscopes. A new extension-fiber theory concerning mitotic mechanism in animals cells. Cytologia 22 193-212 (1957).

5. Nebel, B. R. Personal communication.

6. Longwell, A., and M. Mota. The mitotic spindle and chromosomes as seen under the interference microscope. This report, p. 59.

7. Nebel, B. R., and D. H. Dlesk. Spermatogenic repopulation of mouse testis after X-irradiation. Lancet 274: 1072 (1958).

8. Molè-Bajer, J. Cine-micrographic analysis of c-mitosis in endosperm. Chromosoma 9 332-358 (1958).

9. Ris, H. Chromosome structure. In The Chemical Basis of Heredity, ed. W. D. McElroy and B. Glass. Baltimore: Johns Hopkins Press, 1957 pp. 23-69.

10. Moses, J. M. The relation between the axial complex of meiotic prophase chromosome and chromosome pairing in a salamander (Plethodon cinereus). J. Biophys. Biochem. Cytol. 4 633-638 (1958).

MATHEMATICAL MODELS FOR THE FORMATION AND REGULATION OF POPULATIONS OF FORMED BLOOD ELEMENTS

I. Some Basic Concepts and a First Specific Model

George A. Sacher, Ernesto Trucco, and John H. Borrowman*

This paper discusses some first steps in the development of mathematical models for the maintenance and regulation of populations of blood cells. The basic ideas apply to the erythroid as well as the myeloid lineage.

Broadly speaking, we may distinguish three main physiological activities in the life history of a blood cell, namely those of proliferation, differentiation, and mature function. Several morphological classes or stages of cells have been recognized for each type of blood element, according to various histological and histochemical criteria. These stages form a developmental series, but within the series there is a considerable degree of overlap of the physiological activities, particularly between proliferation and maturation. In our initial model we shall assume that at any given time each cell exists in one of several possible "compartments," but we do not yet attempt to relate these with the "stages" of the hematologist. We shall also assume that there is no overlap of the physiological activities within compartments.

As Patt[1] points out, "the diffuse distribution of the blood forming tissues and the possible variability of the hematopoietic patterns from one locus to another compound the difficulties of evaluation." In our first approach we shall ignore this geographical complication and consider only the case of homogeneous distribution in each compartment.

If N_i ($i = 0, 1, 2, \cdots n$) is the number of cells in the i^{th} stage, our model will be embodied in a system of differential equations specifying the time rates of change, $\frac{dN_i}{dt}$, as functions of $N_0, N_1, N_2, \cdots N_n$. In particular we shall denote by N_0 the number of proliferating cells, and by N_n the number of mature cells, found in the periphery, so that $N_1(t), N_2(t), \cdots N_{n-1}(t)$ will represent intermediate compartments.

In a previous report[2] it was pointed out that a reasonably adequate model cannot consist of linear differential equations only. It is also clear that some feedback control loop should be incorporated into the system in order to maintain and adjust the level of the cell population. In nature these control functions are performed, at least in part, by the humoral substances erythropoietin and leukopoietin, whose chemical structure, properties, and mechanism of action are, however, still imperfectly known.

*Resident Student Associate, Summer, 1958.

Pursuing, then, the ideas set forth in the previous report, we shall take the following differential equation to describe the variation of N_0:

$$\frac{dN_0}{dt} = aN_0 (K_0 - N_0) + bN_0\phi (N_n) - [c_0 + d\psi (N_n)] N_0 - f_0N_0 - \kappa IN_0 \qquad (1)$$

Here a, K_0, b, c_0, d, f_0 and κ are positive constants, ϕ and ψ are functions of N_n to be presently specified. For explanation of I see Equation 1b.

We notice first of all that $\frac{dN_0}{dt}$ is proportional to N_0 itself, so that no production of proliferating cells can occur unless some are already present ("every cell from a cell"). This is a necessary requirement if N_0 is to be truly a primordial stage.

The intrinsic birth term $aN_0 (K - N_0)$ would give rise, if taken by itself, to the ordinary logistic curve for the growth of N_0. A further feedback birth term, namely $bN_0\phi (N_n)$, represents the control exercised by the population of mature cells on the rate of production of new cells. It is reasonable to postulate that $\phi (N_n)$ is a decreasing function of N_n; for simplicity, and only for the time being, we take it to be linear:

$$\phi (N_n) = K_n - N_n \qquad (1a)$$

(K_n being a constant). This assumption is probably true only for a limited range of N_n-values.

The migration term

$$- N_0 [c_0 + d\psi (N_n)]$$

represents the number of proliferating cells that are transformed into the next stage per unit time. Here $\psi (N_n)$ is a second feedback function, again assumed to be linear:

$$\psi (N_n) = K_n' - N_n \qquad (1b)$$

(K_n' = constant).

Finally

$$- f_0N_0 - \kappa IN_0$$

is the death term, f_0 being an ordinary decay constant (intrinsic death), whereas I stands for the intensity of some deleterious external agent, e.g. radiation, since one important purpose of this study is to obtain eventually a quantitative picture of the radiation effects on living cells. As a first

approximation we shall suppose that radiation does not influence the later stages. This would be strictly true, for example, if radiation damage occurred exclusively during mitosis.

For the subsequent stages no birth term will be included, according to our hypothesis that only the first stage is capable of cell division. Thus, the equations for N_1, N_2, $\cdots$ N_n will be of the form

$$\frac{dN_i}{dt} = g_{i-1} - g_i - f_i N_i \qquad (i = 1, 2, \cdots n) \quad , \tag{2}$$

the f_i's being constants.

In these equations g_{i-1} represents immigration from the immediately preceding stage and thus, assuming no migration losses, g_i will be the emigration rate into the next following stage. The quantities

$$- f_i N_i$$

are the intrinsic death terms. We have, of course:

$$g_n = 0 \tag{2a}$$

and, according to Equation 1:

$$g_0 = [c_0 + d\psi (N_n)] N_0 \quad . \tag{2b}$$

For the remaining migration terms we shall assume the simplest possible form, namely

$$\left.\begin{aligned} g_i &= c_i N_i \\ c_i &= \text{constants, } i = 1, 2, \cdots n-1 \quad . \end{aligned}\right\} \tag{2c}$$

In summary, then, the two principal characteristics of this simple model are as follows:

(a) The process of maturation is described as a sequence of <u>irreversible</u> transitions from one maturation stage to the next. We might well assume that (except for the final stage) the death rates are negligible compared to those of emigration, so that

$$f_i = 0 \qquad \text{for } i = 0, 1, 2, \cdots n-1 \quad . \tag{2d}$$

(b) There is essentially only one feedback loop: birth and migration of proliferating cells are regulated by the number of mature cells present.

To avoid cumbersome expressions we shall now put

$$\alpha \equiv b - d \tag{3}$$

$$\beta \equiv aK_0 + bK_n - f_0 - \kappa I - c_0 - dK_n' \tag{4}$$

$$\epsilon \equiv c_0 + dK_n' \tag{5}$$

$$e_i \equiv c_i + f_i \qquad (i = 1, 2, \cdots n-1)$$

$$e_n = f_n \quad . \tag{6}$$

For convenience and simplicity, and because we have practically no data for the individual constants c_i, we shall assume not only that Equation 2d holds, but also that

$$e_1 = e_2 = \cdots = e_{n-1} = c_1 = c_2 \cdots = c_{n-1} \equiv c \quad . \tag{7}$$

The system of Equations 1 and 2, together with 1a, 1b, 2a, 2b, 2c, 2d, and 3 to 7, is then reduced to the following form:

$$\frac{dN_0}{dt} = -N_0 [aN_0 + \alpha N_n - \beta]$$

$$\frac{dN_1}{dt} = \epsilon N_0 - dN_0 N_n - cN_1$$

$$\frac{dN_i}{dt} = c(N_{i-1} - N_i) \qquad \text{for } i = 2, 3, \cdots n-1 \tag{8}$$

$$\frac{dN_n}{dt} = cN_{n-1} - e_n N_n \quad .$$

The number of compartments, i.e., the quantity n, still unspecified, will essentially determine the transfer function, that is, the average time required for a cell to reach complete maturation and, in particular, the distribution of maturation times around that average.

Besides n, the system of Equation 8 still contains seven unspecified constants, namely

$$a, \alpha, \beta, \epsilon, d, c, \text{ and } e_n \quad .$$

To determine them, or at least their orders of magnitude, we can use some information contained in Patt's paper[1] on the steady states of proliferating, differentiating, and mature cells.

Let A_i denote the steady state values of N_i $(i = 0, 1, 2, \cdots n)$. We may, in general, obtain them from the two equations

$$aA_0 + \alpha A_n = \beta \tag{9}$$

$$\epsilon A_0 = dA_0A_n + e_nA_n \quad , \tag{10}$$

since

$$cA_1 = cA_2 = \cdots = cA_{n-1} = e_nA_n \quad . \tag{11}$$

In a plot of A_n vs A_0, Equation 10 represents an equilateral hyperbola, and Equation 9 a straight line of slope $-\frac{a}{\alpha}$ and intercept $\frac{\beta}{\alpha}$ (see Figure 14); the steady states are given by the intersection of these two curves.

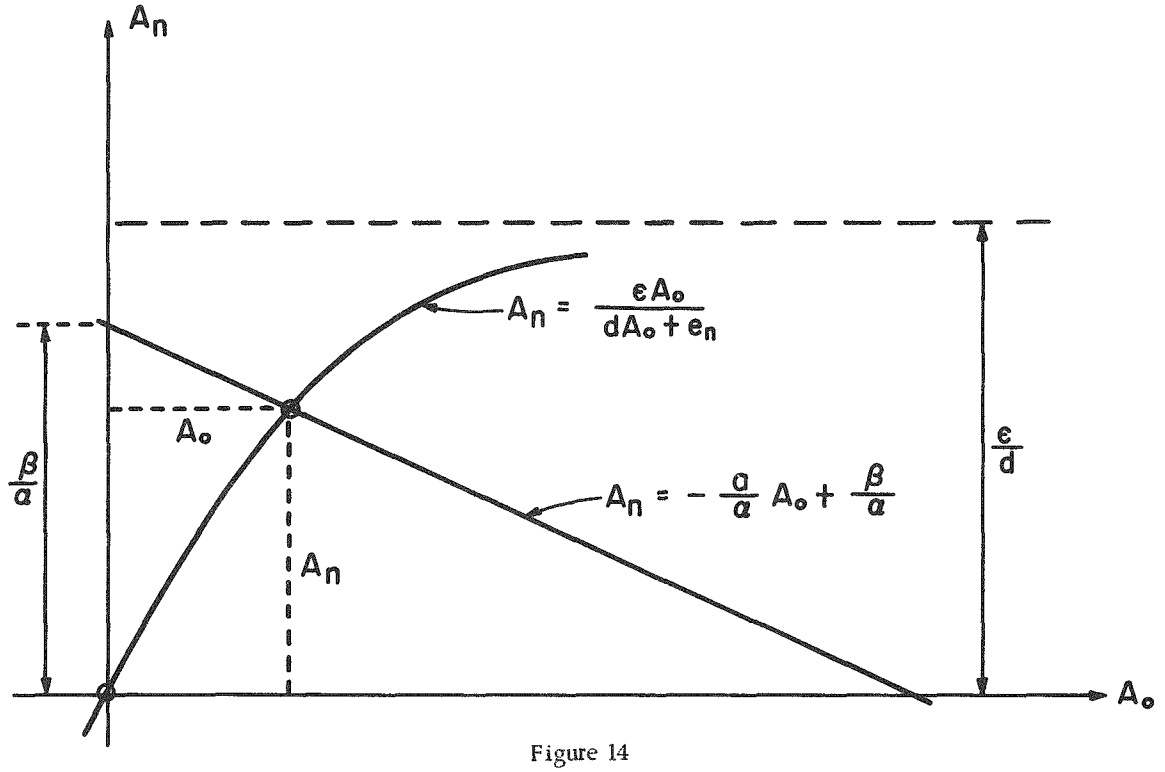

Figure 14

We shall suppose that a, α, and β are positive; the straight line (Equation 9) will then have negative slope and positive intercept. Figure 14 shows that there will be one and only one steady state in the positive quadrant ($A_0 > 0$, $A_n > 0$). (Clearly, negative values of the A_i's are not acceptable.)

In addition, the set of values

$$A_0 = A_1 = A_2 = \cdots\cdots = A_n = 0 \tag{12}$$

always constitutes a possible steady state, as can be seen from Equation 8.

Equation 4 shows that the radiation effect is contained in the parameter β. Our assumption then is that β has a certain positive value, say β_0, in the absence of radiation, and that β decreases to zero as I is gradually increased. Further increase in I will cause β to become negative, in which case the system of Equation 8 will tend to the steady state specified by Equation 12.

From this picture we may obtain a rough estimate of β_0 by experimentally determining the smallest value of κI which destroys practically all of the cells. For simplicity we assume that the quantity κI is time-independent; from available radiobiological information we tentatively adopt the values

$$\kappa \sim 0.01 \text{ r}^{-1} \tag{13}$$

$$\beta_0 \sim 1 \text{ day}^{-1} \quad . \tag{14}$$

Having thus estimated β (or rather β_0), we now turn our attention to the steady-state values in Patt's paper.[1] For the time being we are considering only the case of granulocytes in man.

Patt's Table 1[1] shows that the number of cells produced under stationary conditions is 196.8×10^9 per day. In the steady state this must be equal to any of the quantities which appear in our Equation 11 so that, in particular, we have

$$e_n A_n = 196.8 \times 10^9 \text{ cells per day} \quad . \tag{15}$$

On page 783 of Patt's paper we find that 25×10^9 cells are detectable in the circulation of standard man, but that their number in the entire body outside the marrow is estimated to be about 70 times greater.

Accordingly, we shall take

$$\begin{aligned} A_n &= 70 \times 25 \times 10^9 \\ &= 1{,}750 \times 10^9 \text{ cells} \quad . \end{aligned} \tag{16}$$

From Equations 15 and 16 we therefore obtain e_n:

$$e_n = \frac{196.8}{1{,}750} \approx 0.11 \text{ days}^{-1} \quad . \tag{17}$$

Finally, if we put

$$S(t) \equiv N_1(t) + N_2(t) + \cdots\cdots + N_{n-1}(t) \tag{18}$$

and

$$S_0 \equiv \sum_{i=1}^{n-1} A_i \quad , \tag{19}$$

we may take S_0 to be the over-all steady state value of differentiating cells. Once again we refer to Patt's Table 1; there the number of differentiating cells is given as 512.5×10^9. Thus we may put

$$S_0 = 512.5 \times 10^9 \quad . \tag{20}$$

From that same table we obtain 102.5×10^9 for the number of proliferating cells, so that

$$A_0 = 102.5 \times 10^9 \quad . \tag{21}$$

Furthermore, it is clear from Equations 11 and 19 that we must have

$$A_1 = A_2 = \cdots = A_{n-1} = \frac{S_0}{n-1} \quad , \tag{22}$$

and therefore,

$$c = \frac{e_n A_n}{A_1} = (n-1)\,\frac{196.8}{512.5} \quad . \tag{23}$$

If we put $n = 10$ (a case we have examined), we obtain from Equation 23

$$c = 3.456 \text{ days}^{-1} \quad . \tag{24}$$

We have thus been able to find appropriate values of e_n and c. In the following it will be convenient to introduce the quantities

$$\lambda \equiv \frac{a}{\alpha} > 0 \tag{25}$$

and

$$\mu \equiv \frac{\epsilon}{dA_n} > 1 \quad . \tag{26}$$

The remaining four constants, a, α, ϵ, d, can now be obtained from Equations 9 and 10 in terms of the known quantities A_0, A_n, β_0, e_n, and the two unknown parameters λ, μ. We have, in fact:

$$\alpha = \frac{\beta_0}{\lambda A_0 + A_n} \quad , \tag{27}$$

$$a = \lambda\alpha \quad , \tag{28}$$

$$d = \frac{e_n}{(\mu-1)A_0} \quad , \tag{29}$$

$$\epsilon = d\mu A_n \quad . \tag{30}$$

It appears, therefore, that, utilizing Patt's experimental data, the main properties of our differential Equations 8 can be studied, provided we assign values to the three unknown parameters n, λ and μ. In practice, of course, we shall let the parameters vary, and investigate under what circumstances the model will correctly simulate the experimental situations. Some of the features we should require of our system have been mentioned in the previous report.(2)

We have begun the parameter study, using the PACE analog computer available at the ANL Applied Mathematics Division. We are greatly indebted for help and advice to that Division, in particular to Dr. N. F. Morehouse and his group.

Our main concern at this time is in determining the existence of a stable steady state for a given set of parameters and for all positive values of $\beta \leq \beta_0$.

Perhaps the most accurate way of dealing with this problem would be to linearize the Equations 8 in the neighborhood of a steady state, and then investigate the characteristic equation; we find, however, that for our purposes we obtain sufficient information by solving the nonlinear equations directly on the computer.

Preliminary studies with n = 2 and n = 10 indicate that the required steady state does not always exist. The system of Equation 8 will in many cases perform undamped oscillations of large amplitude (limit cycles).

This is not unexpected: the presence together of a large feedback birth term and a considerable time delay between birth and maturity (both indicated by experimental and clinical evidence) will cause any such system to be at best conditionally stable, i.e. stable only for certain values of the parameters. We may expect to find that stabilization of the present model system can be achieved only for cases in which some of the parameter values are unrealistic. The behavior of the present model and of modifications will be discussed in future communications.

References

1. Patt, H. M., A consideration of myeloid-erythroid balance in man. Blood 12: 777-787 (1957).

2. Sacher, G. A., Progress Report: Gamma Ray Toxicity Program. III. Mathematical Analysis of the Behavior of Cellular Systems. Quarterly Report of Biological and Medical Research Division, Argonne National Laboratory. ANL-5486 (1955) pp. 47-52.

UPTAKE OF MICRONUTRIENT ELEMENTS BY SETARIA IN RELATION TO AVAILABLE BORON

Wayne J. McIlrath,* Jan A. DeBruyn,* and John Skok

The level of available boron has been reported[1] to influence the uptake and accumulation of various other mineral elements in dicotyledonous plants. We have been concerned with observed differences between dicotyledonous and monocotyledonous plants with respect to boron requirements and to the distribution and chemical form of boron in various cellular fractions. This report describes experiments carried out to determine the effect of boron on the uptake of four micronutrient elements (Fe, Cu, Mn and Mo) in a monocotyledonous plant, the grass Setaria spacelata.

Plants were grown in quartz sand and were supplied with complete nutrient solutions containing either 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 100, or 200 ppm boron. They were harvested after a growth period of about seven months. Spectrographic analyses of boron and the other four mentioned micronutrient elements were made on replicated ash samples prepared from oven-dried plant shoots.

The plants receiving no boron showed no noticeable boron deficiency symptoms. Such symptoms can be easily obtained within a few days in most dicotyledonous plants grown in sand. However, grasses are known to have a very low requirement for boron, and the sand apparently contained sufficient boron as a contaminant to supply needs for growth in this plant. Slight visual evidence of toxicity symptoms occurred at 5 ppm boron but these were not accompanied by significant reduction in dry weight. Marked toxicity and reduction of dry weight occurred at all levels over 10 ppm boron.

Within the nontoxic ranges, the level of available boron had no significant effects on total dry weight, ash content, or content of micronutrient elements (excluding boron itself). At the highly toxic boron levels, reduction in dry weight was accompanied by an increase in percentage ash.

The effect of variation in available boron on the micronutrient element content, then, differs in monocots and dicots; while it has been shown to result in pronounced changes in several dicotyledonous plants, no such effects were produced in Setaria.

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We wish to thank J. K. Brody and J. A. Goleb of the Chemistry Division for performing the spectrographic analyses.

Reference

1. Gauch, H. G. and W. M. Dugger, Jr. The physiological action of boron in higher plants: A review and interpretation. Maryland Agr. Expt. Sta. Bull. A-80 (1954).

PHOTOPERIODIC BEHAVIOR OF SUNFLOWER

John Skok, Hubert J. Dyer* and N. J. Scully

Comparatively little detailed information has been reported on photoperiodic responses of sunflower, and flowering in this plant has been generally thought to be independent of day length. Elongation of sunflower stem was found by Downs et al.[1] to be regulated by the same photoreaction, i.e., photoreversibility by red and far-red light, that is responsible for elongation of stems of numerous other plants, as well as for the control of many other light-dependent responses including photoperiodic control of flowering. It was observed independently here and at Brown University that sunflower responds to photoperiod and appeared to be of the short-day type. The response has therefore been investigated in more detail in a cooperative study carried out at both laboratories

Mammoth Russian sunflower plants were grown under various photoperiods and under treatments in which the daily dark periods were interrupted with light periods Data on the flowering response were obtained by recording the presence of macroscopic flowers or flower buds, and also by microdissection of the terminal buds to determine effects on initiation of floral primordia. The vegetative meristem of sunflower is perfectly flat, the earliest noticeable morphogenic change associated with flowering is a mounding of the flat meristem. The mounding then continues and is accompanied by considerable enlargement; finally individual flower primordia are produced.

The results of all the experiments confirmed our impression that sunflower is a short-day type of plant It will flower under a wide range of photoperiodic conditions, including excessively long days (as long as 20 hr) and even a regime in which the daily dark periods are interrupted by one-hour light periods, if it is permitted to grow for a sufficiently long period. However, short photoperiods promote flowering, both by hastening the initiation of flower primordia and by hastening the development of primordia into macroscopic floral structures. Interruption of the dark periods by light is effective in inhibiting both the initiation of flower primordia and the development of primordia into macroscopic floral structures.

In sunflower there is not only an unusually wide range of photoperiodic conditions under which flowering may take place, but also an unusual amount of variability in the time of flowering among individual plants in a given population. This may be related to a maturation requirement (vegetative growth requirement for flowering),

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pointed out by Habermann and Wallace,[2] which must be met before flowering can occur.

References

1. Downs, R. J., S. B. Hendricks, and H. A. Borthwick. Photoreversible control of elongation of pinto beans and other plants under normal conditions of growth. Botan. Gaz. 118 199-208 (1957).

2. Habermann, H. M. and R. H. Wallace. Transfer of flowering stimulus from stock to scion in grafted Helianthus annuus. Am. J. Bot. 45 479-482 (1958).

THE MITOTIC SPINDLE AND CHROMOSOMES AS SEEN UNDER THE INTERFERENCE MICROSCOPE

Arlene Longwell* and Miguel Mota*

The mitotic spindle has always been the most mysterious entity of mitosis although its importance has long been recognized. Because it cannot be stained with any of the dyes commonly used in cytology it has been called the achromatic spindle. The presence and exact shape of the spindle has always been more or less poorly defined and its boundaries have been difficult to ascertain All of this resulted in a great controversy which included even the hypothesis that the so-called achromatic spindle did not exist. Mazia and Dan,[1] however, by isolating the mitotic apparatus (spindle, asters and chromosomes) in sea urchin eggs conclusively demonstrated the existence of the achromatic spindle and contributed some knowledge of its chemical composition.

Chromosomes, on the other hand, have great affinity for acid dyes (a property from which they derive their name) and in general present no problem from this point of view. Although much remains to be investigated, the morphology of the chromosomes is known in considerable detail. Knowledge of their number, length, constrictions, points of chiasma, and so forth, enables us to study their behavior with greater facility than is possible for the spindle.

Of the more recent tools used in cytology, the phase contrast microscope resulted in very little new information concerning the mitotic spindle. However, it demonstrated the shape of the space occupied by the spindle, especially in living cells, by showing the mitochondria arranged around it. Unstained chromosomes as seen with the phase microscope do not differ essentially from the picture obtained in the ordinary microscope with stained preparations. The polarization microscope, with the refinements introduced by Inoue, has been valuable in showing that the spindle presents a positive birefringence and is, therefore, composed of oriented molecules. The interference microscope, a relatively recent development, presents new possibilities.

With the interference microscope, small differences in the dry mass of parts of individual cells show up as differences in color when white light is used, and as differences in intensity when monochromatic light is used. This is a consequence of the relationship between the concentration of the solids in the cell and the phase changes undergone by the light which passes through the object. The two-beam interference microscope operates to translate the invisible phase changes to differences in intensity or in color.

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This is accomplished by the optical formation of an interference effect between two beams, one of which has not passed through the object and the other of which has passed through the object and has been modified by it.(2) The phase difference between the two interfering beams can be varied, enabling one to measure the optical path differences of the light through the object, as compared to the mounting medium. By varying the phase difference between these two beams it is possible to choose the type of contrast, color or intensity best suited for a study of the particular detail being observed. The color or intensity of a particular point in a cell as seen in the interference microscope is dependent on the difference between the product of the refractive index and thickness of that region on the one hand, and the refractive index of the mounting medium on the other hand. By varying the refractive index of the mounting media, contrast within the cell can be greatly altered so as to show a maximum or a minumum degree of detail either in the nucleus or in the cytoplasm. The usefulness of the interference microscope in the study of the mitotic spindle has already been shown by Mitchison and Swann(3) and Rustad (4)

Meiosis was studied in wheat, Tradescantia, corn and grasshopper. The spindle is described for plant material. The material was fixed in Carnoy's solution and mounted in acetic acid in concentrations ranging from 45% to 5.0%, and in distilled water. Other mounting media and living material are being investigated and will be reported on later. Both the Cook-Dyson and the A-O Baker interferometers were used. The following is a preliminary report; color micrographs, optical path retardation and dry mass estimates will be included in a more detailed report on the complete study.(5)

From mid- to late prophase of the first meiotic division in wheat microsporocytes, the nucleus appears surrounded by a differentiated zone probably similar to the "clear zone" described by Bajer(6) in living endosperm cells. It shows a higher specific mass than the rest of the cytoplasm and seems to grow until later prophase or prometaphase when it breaks down. The later the stage of prophase, the closer the ring appears to the nuclear membrane. There is no concentration gradient between the nuclear membrane and this dense ring as would be expected should this material be coming from the nucleus It is possible then that the material is cytoplasmic in origin, and that it approaches and touches the nuclear membrane in late prophase because of growth of the ring and growth of the nucleus and possibly even because of movement of the ring towards the nucleus. In pro-metaphase I in corn and wheat, the chromosomes lie in a mass of higher density than the rest of the cytoplasm, and only remnants are evident of the dense ring which at earlier stages had surrounded the nucleus. It is possible that the material forming the ring gives rise to this mass of higher density, or at least makes a substantial contribution

to it, and that the spindle thus arises from this region of increased mass. There is no evidence of fibers in the dense pro-metaphase region of the cytoplasm.

When the chromosomes arrange themselves in the equatorial plate, the spindle appears composed of the spindle fibers and a more uniform mass in which these are imbedded. The density of this mass is slightly lower than that of the cytoplasm. The fibers or groups of fibers, on contrary, show a considerably higher density than the cytoplasm, and appear double; some of them are attached to the chromosomes.

In corn, where the chromosomes are relatively small compared to the size of the cell, the spindle at late metaphase or in mid-anaphase shows two poles at either side of the equatorial plate, as if a shorter and a longer spindle were superimposed. The chromosomes seem to reach only the nearer pole and do not move on to the farthest pole. In late anaphase the region between the two groups of daughter chromosomes also shows fibers. The region between these fibers is equivalent in density to the cytoplasm. The fibers are clearly denser, almost approaching the density of the chromosomes. From this stage to late telophase the density of the region between the two daughter nuclei increases until it greatly exceeds the density of the rest of the cytoplasm In the equator of this region a thin band of still higher mass develops later to form the new cell wall. This increase in mass in the region between the two anaphase groups is in good agreement with the fact that in some cases ribonucleoproteins have been detected in that region after anaphase [7] Should this increase in mass result from material released from the chromosomes, it would support the hypothesis that the movement of the chromosomes in anaphase results from the release of some substance from the chromosomes.[8]

By increasing the refractive index of the medium until it approaches that of the cytoplasm, it is possible to make the cytoplasm appear practically invisible, and the chromosomes appear much better defined. In Tradescantia the major coil is clearly seen, and the spindle attachment regions are well defined and quite conspicuous. Wheat chromosomes appear the same as in stained preparations, showing that no important artifact is introduced by staining. In grasshopper spermatocytes the four chromatids in diplotene are so clearly seen that crossing over can be analysed at least as well as in stained preparations. Metaphase chromosomes are also seen as in stained preparations with the advantage over the phase microscope that they appear in color different from that of the surrounding medium, making differentiation much clearer.

References

1. Mazia, D. and K. Dan. The isolation and biochemical characterization of the mitotic apparatus of dividing cells. Proc. Natl. Acad. Sci. 38 826-838 (1952).

2. Barer, R. Phase contrast and interference microscopy in cytology. In: Physical Techniques in Biological Research, ed. G. Oster and A. W. Pollister, New York: Academic Press, Inc., 1956. Vol. 3, pp. 29-90.

3. Mitchison, J. M. and M. M. Swann. Measurements on sea urchin eggs with an interference microscope. Quart. J. Microscop. Soc. 94 381-389 (1953).

4. Rustad, R. R. An interference microscopical and cytochemical analysis of local mass changes in the mitotic apparatus during mitosis. Exptl. Cell Res. 16 575-583 (1959).

5. Longwell, A. C., and M. Mota. Relative distribution of mass in dividing cells by interference microscopy. Endeavour, in press.

6. Bajer, A. Cine-micrographic studies on mitosis in endosperm III. Exptl. Cell Res. 13 493-502 (1957).

7. Jacobson, W. and M. Webb. The two types of nucleoproteins during mitosis. Exptl. Cell Res. 3 163-183. (1952).

8. Mota, M. A new hypothesis of the anaphase movement. Proc. Internat. Gen. Symp., 1956. Tokyo: The Science Council of Japan, 1957. pp. 113-116.

THIRTY-DAY SURVIVAL OF FEMALE MICE AND RATS GIVEN SINGLE, WHOLE-BODY EXPOSURES TO FISSION NEUTRONS

D. L. Jordan and H. H. Vogel, Jr.

Several experiments have been carried out over the past 2 years to ascertain the $LD_{50/30}$ of CF No. 1 female mice given whole-body exposures to fission neutrons at the CP-5 reactor. A similar study was made with Sprague-Dawley female rats. The results are reported here.

Figure 15 shows the probit regression of mortality on dose for 474 mice exposed to a dose range of 276-375 rads of fission neutrons at a dose rate of 7-8 rads per min. The $LD_{50/30}$ was 323 $\pm$ 5 rads. Table 12 gives the details of these exposures.

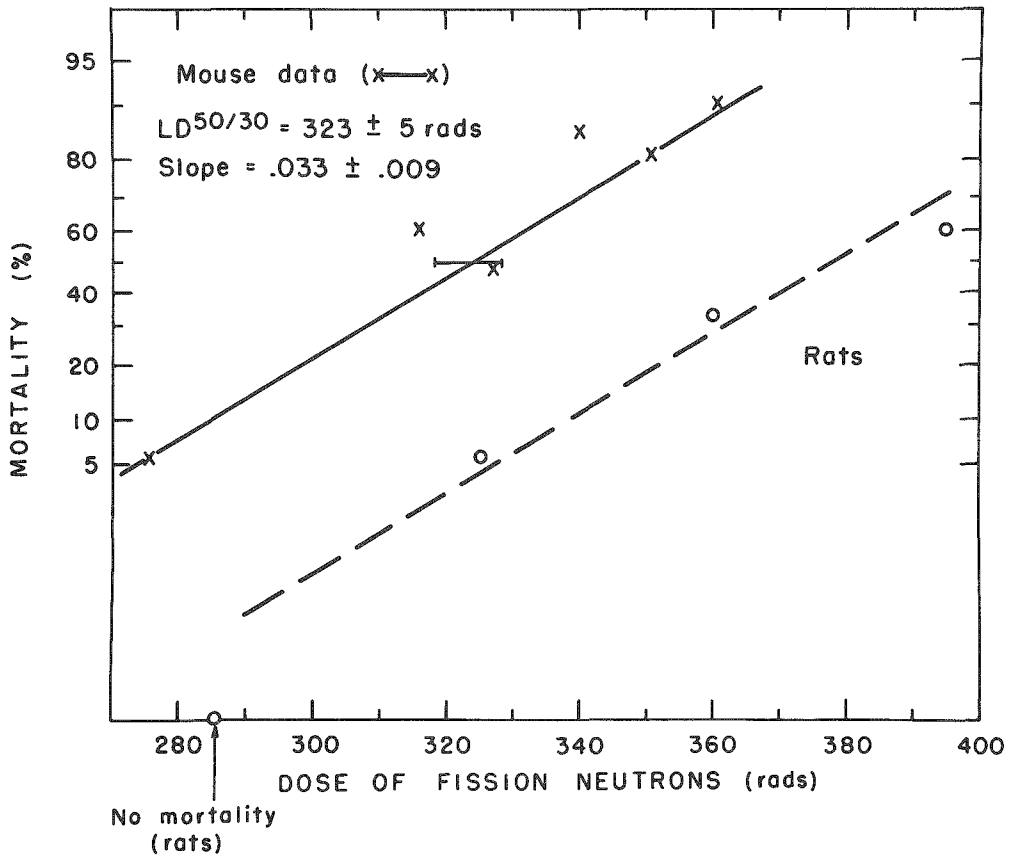

Figure 15. Effect of dose on cumulative mortality in neutron irradiated mice and rats.

The data in Fig. 15 and Table 13 indicate that the 30-day median lethal dose for the rats was approximately 380 rads.

TABLE 12

Mortality after single exposures
of female CF No. 1 mice to fission neutrons
at CP-5. Dose rate = 7-8 rads/min. (Reactor at 2000 kw.)

Total dose, rads	Time of exposure, min	No. of mice exposed	30-day mortality	
			No. dead	%
276	48	36	2	5.8
316	42	38	23	60.6
327	40-65	171	83	48.5
340	45-48	35	30	85.7
351	47-55	86	70	81.4
361	46-60	52	47	90.4
375	48-60	56	56	100.0

TABLE 13

Single exposures of female Sprague-Dawley rats to fission
neutrons at CP-5. Dose rate = 3.6 rads/min. (Reactor at 1000 kw.)

Total dose, rads	Time of exposure, min	No. of rats irradiated	30-day mortality		Mean survival time of 30-day survivors,** days
			No. dead	%	
216	60	18	0	0	407
288	80	18	0	0	436
324	90	18	1	5.5	470
360	100	18	6	33.3	412
396	110	18	11	61.1	460
Unirradiated controls		10*	-		

*2 of 10 unirradiated controls still alive 920 days after exposure date. Mean after survival time will exceed 810 days.

**33 of 70 rats, surviving past 30 days, had large mammary tumors (sometimes multiple). All the neutron-irradiated rats had complete (Grade IV) cataracts within 1 year.

RADIATION PROTECTION EXPERIMENTS IN MICE EXPOSED TO FISSION NEUTRONS

II. Combination Therapy with Cysteine Preirradiation and with Bone Marrow Cells and Streptomycin Postirradiation

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

The additive and perhaps synergistic protective action of intravenous bone-marrow cells and the antibiotic streptomycin has been demonstrated for CF No. 1 female mice exposed to single lethal doses of fission neutrons.[1,2] Previous experiments from this laboratory indicated that pretreatment of neutron-irradiated mice with cysteine effectively reduced the radiation dose by approximately 7-8%.[3] It seemed probable, therefore, that the three agents in combination might produce even better protection than the two used previously, and should allow experiments at doses in the supralethal range.

A preliminary experiment was carried out to test the effectiveness of cysteine given intraperitoneally rather than intravenously as in all our previous work.[3] Since the bone marrow cells are most effective when injected into a vein after irradiation, it was hoped that another route could be used for the cysteine injection before neutron exposure. The results of this experiment (Figure 16) indicated that the cysteine given by this route

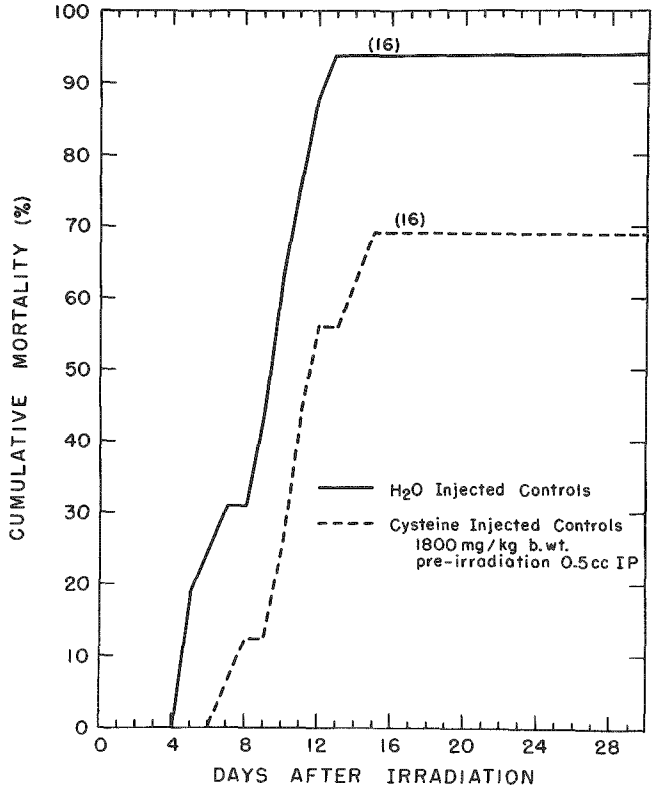

Figure 16

The effect of cysteine on cumulative mortality in neutron-irradiated mice. The radiation dose was 350 rads. Figures in parenthesis represent numbers of animals.

was effective. Mice given 0.5 cc of a 10% cysteine solution (pH 7-8, 1800 mg/kg body weight) exhibited apparent dose reductions in this same range as previously reported.*(3)

The three treatments were therefore combined as follows. Cysteine was injected intraperitoneally before neutron exposure. These irradiated animals were then given 0.1 cc of bone marrow cells (from other female mice of the same strain), by injection into a tail vein within a few hours after irradiation; the bone marrow technique was that described in a previous report.(1) The mice were also given a daily dose of 0.5 cc streptomycin subcutaneously for a 10-day period after exposure.

Other neutron-irradiated mice received an injection of water as a preirradiation control for the cysteine. After irradiation these animals received 0.1 cc Tyrode's solution (the bone marrow vehicle) and daily injections of 0.5 cc saline solution. This group constituted the neutron-irradiated, untreated controls.

Data for a series of 4 experiments are summarized in Table 14 and illustrated graphically in Figure 17. It is clear from these data that we are dealing with supralethal doses of fission neutrons, since only 1 of the control mice (out of 53) survived the lowest exposure, and all the controls

TABLE 14

Effects of combination therapy (cysteine, bone marrow cells, and streptomycin) on 30-day mortality in mice exposed to supralethal doses of fission neutrons*

Experiment	Duration of irradiation, min	Total dose, rads	Neutron-irradiated control mice		Neutron-irradiated treated mice	
			No. dead / No. exposed	%	No. dead / No. exposed	%
A	50	335	31/32	96	1/32	3
B	50	335	8/8	100	1/16	6
C	55	370	7/7	100	1/16	6
D	60	400	6/6	100	3/16	18
Four experiments combined	50-60	335-400	52/53	98	6/80	7.5

*Exposures to fission neutrons were carried out at the CP-5 reactor, operating at 2000 kw. The dose rate was ~6.7 rads/min.

*We should like to thank Dr. Robert Straube for assistance in these cysteine dosage experiments.

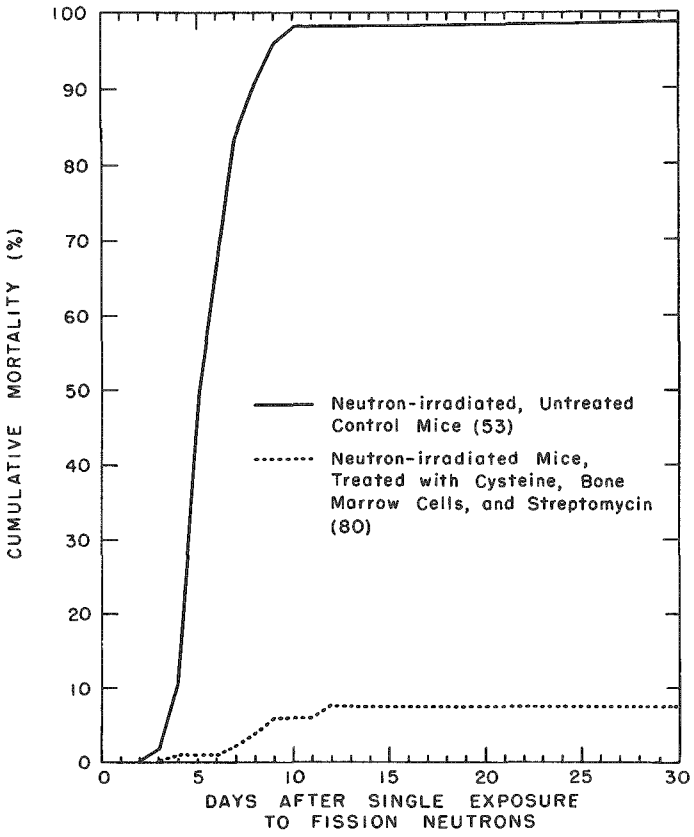

Figure 17

Comparison of cumulative mortality, in the acute 30-day period following single exposure, between neutron-irradiated control mice and neutron-irradiated mice treated with cysteine, bone marrow, and streptomycin injections. The dose range was 335-400 rads. This figure presents the combined results of the four experiments in Table 14. Figures in parenthesis represent numbers of animals.

died in the last three experiments. Under the experimental conditions at the CP-5 reactor, an exposure of approximately 350 rads will kill at least 95% of irradiated mice. Certainly the exposures C and D (370 and 400 rads) are well above the $LD_{99/30}$ dose level. It is evident also from the results of these experiments that the treated mice are well protected. Only 6 out of 80 mice (7%) treated with the triple technique described above, died within the acute 30-day period. It is interesting to point out that, in both experimental and control groups, all the mortality in the acute period took place in the 10-12 days after exposure.

There is some evidence that as the dose is increased, the protection becomes less effective: half of the treated mice that died were in the highest (400 rad) exposure group. Further experiments will be carried out at even higher doses.

Figures 18 and 19 give the body weight data for the various treated groups. Figure 18 illustrates the data for experiment A (Table 14), while Figure 19 compares the weights of the mice in the other three experiments, B - D. In all 4 groups there was a sharp decline in body weight for at least 4 days following exposure, but all of the treated groups exhibited definite recovery with gains in average body weight, usually beginning between the 6th and 7th days after exposure. This is evidence, we believe, that the introduced bone marrow cells have successfully been transplanted

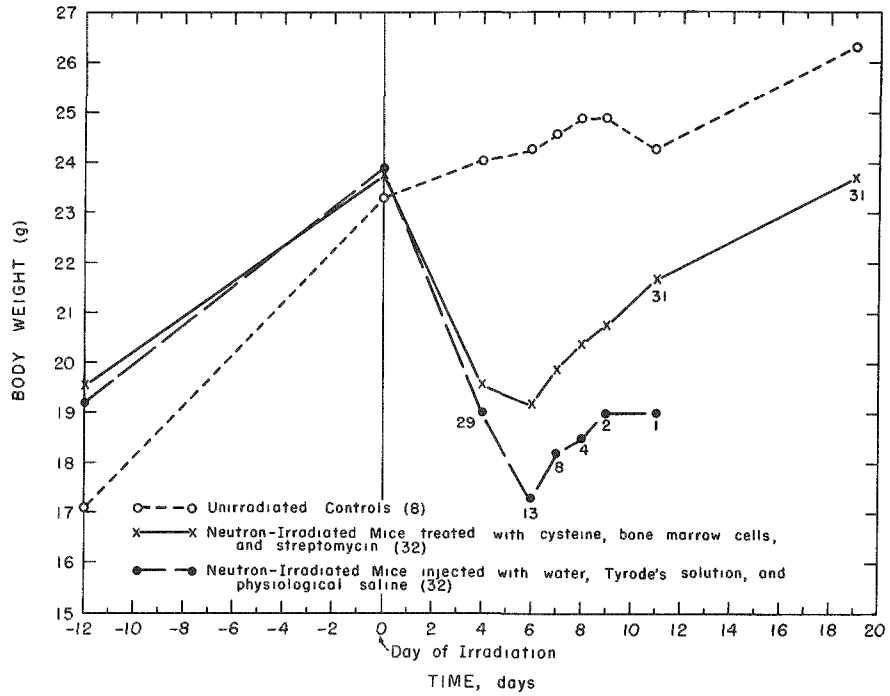

Figure 18. Comparisons of body weight between unirradiated control mice, neutron-irradiated controls, and neutron-irradiated mice "protected" with cysteine, bone marrow cells, and streptomycin in combination. Figures below the lines indicate the number of mice alive at that particular day; figures in parenthesis represent numbers of animals.

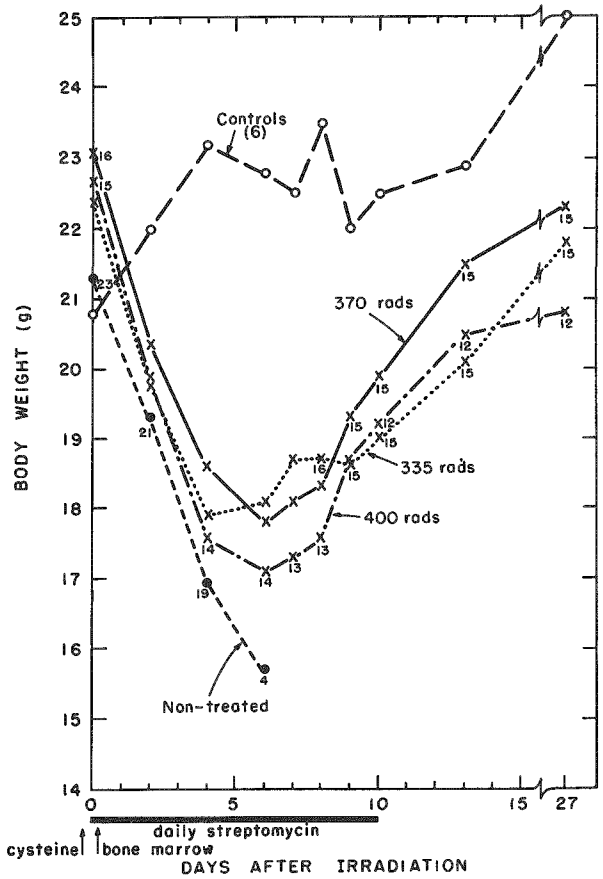

Figure 19

Comparisons of body weight in three groups of neutron-irradiated protected mice (Groups B, C, D) compared with untreated irradiated and unirradiated controls (compare with data in Figure 18 and Table 14). Figures in parenthesis represent numbers of animals.

into the host's blood-forming system and are stimulating hematopoietic recovery. However, cumulative mortality curve for untreated neutron-irradiated mice (Figure 17) shows that at that time, one week after exposure, more than 80% of the mice are dead. This suggests why bone marrow cells alone are not very effective in mice exposed to lethal doses of fission neutrons.[1] The antibiotic streptomycin helps to protect the mice from fatal bacteremia and septicemia during the 10-day period following exposure.[4] By this time the bone marrow, ineffective against the intestinal syndrome, has taken over its protection of the mouse hematological system. The cysteine helps the entire protective process by reducing the total effective radiation dose by approximately 10%.

References

1. Vogel, H. H., Jr. and D. L. Jordan. Radiation protection experiments in mice exposed to fission neutrons. Semiannual report of Biological and Medical Research Division, Argonne National Laboratory. ANL-5841 (1958) pp. 116-120.

2. Vogel, H. H., Jr. and D. L. Jordan. The protective action of bone marrow cells combined with antibiotic therapy in mice irradiated with fission neutrons. Paper read at International Congress of Radiobiology, Burlington, Vermont (1958). Abstract Radiation Research: 9 199 (1958).

3. Patt, H. M., J. W. Clark, and H. H. Vogel, Jr. Comparative protective effect of cysteine against fast neutron and gamma irradiation in mice. Proc. Soc. Exptl. Biol. Med. 84 189-193 (1953).

4. Hammond, C., H. H. Vogel, Jr., J. W. Clark, D. B. Cooper, and C. P. Miller. The effect of streptomycin therapy on mice irradiated with fast neutrons. Radiation Research 2 354-360 (1955).

PRELIMINARY TESTS ON THE ACTIVITY OF CERBARTROL AS A POSSIBLE PROTECTIVE AGENT AGAINST RADIATION EFFECTS IN MICE

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

Cerbartrol ® is a protein-free and fat-free bone marrow extract combined with malic acid.* Clinical tests showed that it has some therapeutic value in rheumatoid arthritis and osteoarthritis.[1,2] Preliminary studies at the University of Bologna,[3,4] suggested that the extract might be of some value in increasing radioresistance in mammals.

Two preliminary experiments were therefore undertaken. In the first, 32 mice were irradiated with 350 rads of fission neutrons. Of these, the 8 untreated controls all died within 21 days. The remaining 24 irradiated mice were divided into 3 groups and injected intramuscularly with Cerbartrol once daily for 1 week after exposure, at a dose of 0.05 cc, 0.1 cc or 0.2 cc.** There was no evidence of protection. A second, similar experiment was carried out in mice exposed to 1068 rads Co^{60} γ-rays, at 10 rads/min, using doses of 0.1 and 0.2 cc. Results of this experiment were also completely negative: all mice died within 15 days.

References

1. Lucherini, T. G. Tuccimei, Minerva Medica 2 (II) 60 (1951) cited in (3).

2. Smith, L. W. Medical Times 83: 851 (1955) cited in (3).

3. Report on the pharmacologic activities of Cerbartrol, prepared by Prof. Aldo Cestari, Director of the Institute of Pharmacology, University of Bologna, Italy. (Unpublished)

4. Biological action of Cerbartrol corticoid and cortico-tropic activity. Dr. A. Castelli and Dr. A. M. Sechi, Biochemistry Institute, University of Bologna, Italy. (Unpublished)

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*We thank the Cerbini Research Corporation, New Rochelle, N.Y., for supplying the Cerbartrol for these experiments. The material was either in the form of 1 cc ampuls for injection or in the form of pills to be taken orally.

**The material was supplied in the form of 1 cc ampuls. The concentration was not given. Doses here were based on information supplied by the company indicating that 1 cc/day represented an "average human dose."

RADIATION RECOVERY

V. Recovery in Irradiated Mice at One and Two Months after Exposure to Co^{60} γ-Rays or Fission Neutrons

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

During the past several years, the Neutron Toxicity group has studied recovery in CF No. 1 female mice after exposure to γ-rays and to fission neutrons. Previously we have reported the results of experiments using paired equal doses and varying intervals between exposures: a few minutes,[1] a few hours,[2] or 10 to 15 days.[3,4]

The experiments reported herein were carried out using this same split-dose technique, but extending the time between exposures to 1 and 2 months.

Co^{60} γ-Rays

Twenty-eight Days between Exposures. The animals used were young adult CF No. 1 females, weighing 18 to 25 g. One group was irradiated with 300 rads of Co^{60} γ-rays, while another was exposed to 600 rads. One month (28 days) later these 2 groups, together with 2 groups randomly selected of unirradiated age-controls from the same shipment, were exposed to 850 rads of Co^{60} γ-rays. This exposure should kill less than 50% of the mice in 30 days when delivered as a single whole-body exposure, and, actually, approximately 40% of the mice of each age-control group died in the acute 30-day period (Figure 20). The mice that had been irradiated 28 days previously with 600 rads all died during the 30 days after their second exposure to 850 rads. The 300-rad group showed a 30-day mortality of more than 80%, significantly elevated from that of the single-irradiated age-control groups (Figure 20).

It is clear from this experiment that part of the first dose is still residual (i.e., recovery is not 100% complete) 28 days after γ-ray exposure when the first dose is approximately one-third to two-thirds of the $LD_{50/30}$ dose (900 rads Co^{60} γ-rays).

Sixty Days between Exposures. In this case, 2 groups were irradiated with 596 rads Co^{60} γ-rays (approximately 2/3 $LD_{50/30}$ dose) and 60 days later these mice, together with 2 groups of age-controls, were exposed to a second dose of 890 or 930 rads, (approximately the $LD_{50/30}$ and $LD_{80/30}$, respectively, for single exposures to CF No. 1 female mice). It was estimated that if even 5% of the dose from the first exposure was still residual after the two-month period, 100% of the mice should die. The

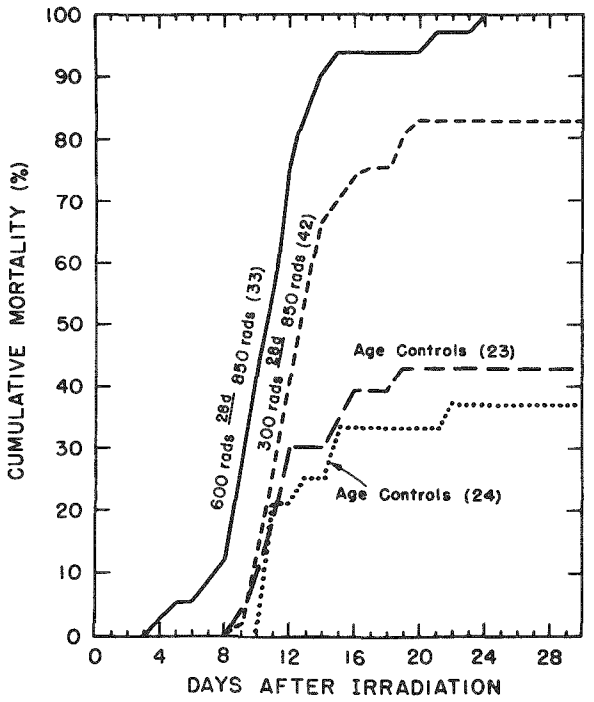

Figure 20

Cumulative mortality of CF No. 1 female mice irradiated with 850 rads Co^{60} γ-rays, 28 days after previous exposure to either 300 rads or 600 rads. Compare mortality for each dose group with that of its age-control groups, i.e., the mice receiving merely the single exposure of 850 rads; the dashed line represents the age-controls for the 300-rad group, the dotted line the age-controls for the 600-rad group. Figures in parentheses give numbers of animals per group.

results of this experiment (Figure 21) indicate no significant difference in 30-day mortality between the irradiated mice and their age-controls. We may conclude, therefore, that no significant portion of the first γ-ray dose (596 rads) is residual after a 2-month period, at least as measured by 30-day mortality.

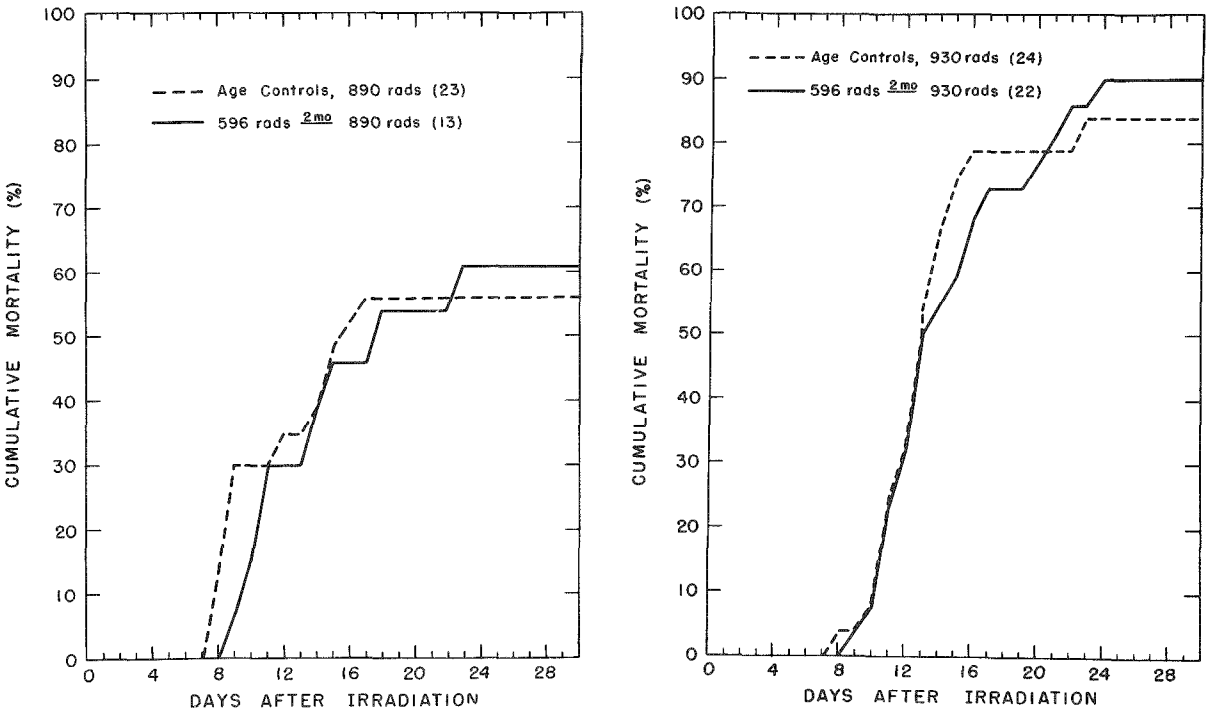

Figure 21. Cumulative mortality of CF No. 1 female mice irradiated with Co^{60} γ-rays: 890 rads (A) or 930 rads (B), 2 months after a preliminary exposure to 596 rads. Each group is compared with its own age control which received only the single exposure of either 890 or 930 rads. Figures in parentheses give numbers of animals per group.

Fission Neutrons

Thirty-three Days between Exposures. In Figure 22 are compared the cumulative mortality data for two groups of mice exposed to fission neutrons. The experimental group received 160 rads exposure, followed 33 days later by a second exposure of 340 rads. These should be compared with the mortality of the age-control group of 32 mice, irradiated only once with 340 rads. The second exposures for the two groups were done simultaneously. In each group only one mouse survived the acute period (97% deaths in 30 days). There is no evidence from this experiment that any residual dose remains from the first 160 rads of fission neutrons after an interval of 33 days.

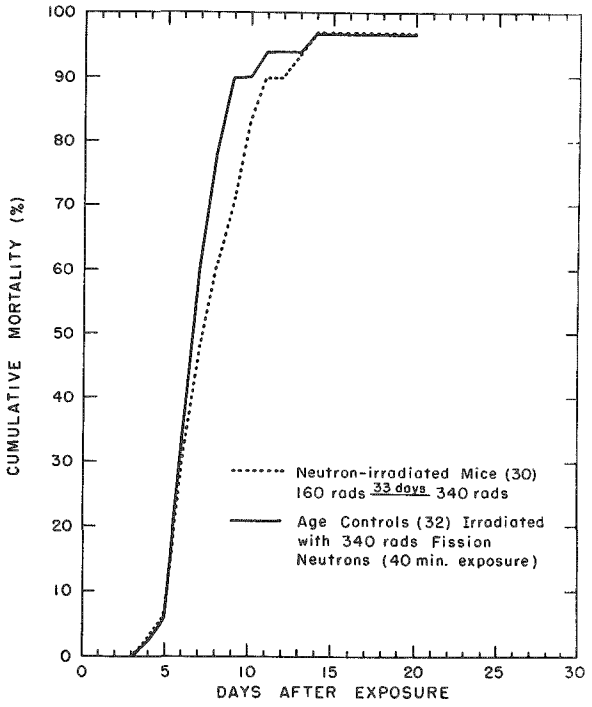

Figure 22

Cumulative mortality of CF No. 1 female mice irradiated with 160 rads fission neutrons (approximately one-half $LD_{50/30}$ dose) and, 33 days later, exposed to a second dose of 340 rads. Compared with age controls exposed to a single dose of 340 rads fission neutrons. Figures in parentheses give numbers of animals per group.

Sixty Days between Exposures. Figure 23 summarizes the mortality data from a similar exposure to split doses of fission neutrons, carried out at the CP-5 reactor, with an interval of 2 months between exposures. Mice exposed to 100 rads or 185 rads 60 days prior to a second exposure to 300 rads of fission neutrons showed no increase in 30-day mortality when compared to a group of age controls simultaneously exposed to a single dose of 300 rads. There is even an indication (since the mortality in both split-dose groups is lower than that in the age controls) that the first dose of neutrons might be slightly "beneficial" as measured by 30-day mortality. At any rate, we may conclude that 60 days after a first neutron dose (from 30% to 57% of the $LD_{50/30}$ single whole-body dose) there seems to be no residuum as measured by the 30-day acute mortality.

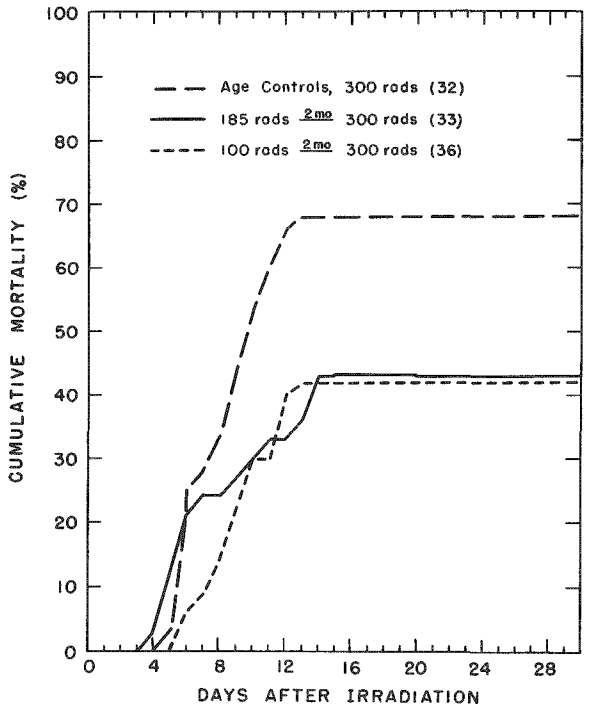

Figure 23

Cumulative mortality of CF No. 1 female mice irradiated with 300 rads fission neutrons, 2 months after a preliminary exposure of 100 rads or 185 rads of neutrons. Compared with age control group which was irradiated only once with 300rad dose. Figures in parentheses give numbers of animals per group.

References

1. Vogel, H. H., Jr., V. Johnson, N. Bink, and R. Barhorst. Radiation recovery. II. Variation of the time interval between equal paired doses of Co^{60} gamma radiation. Biological and Medical Research Division Quarterly Report. Argonne National Laboratory, ANL-5655 (1956). pp. 96-99.

2. Vogel, H. H., Jr., and D. L. Jordan. Radiation recovery. III. Time intervals of several hours between equal paired doses of Co^{60} gamma radiation. Biological and Medical Research Division Quarterly Report. Argonne National Laboratory, ANL-5696 (1956). pp. 77-80.

3. Vogel, H. H., Jr., J. W. Clark, and D. L. Jordan. Recovery after fission neutrons: Paired equal doses with varying time intervals. Radiation Research 7 458-459 (1957) (Abstract).

4. Vogel, H. H., Jr., J. W. Clark, and D. L. Jordan. Preliminary report: Recovery from radiation. I. The effect of paired equal doses of Co^{60} γ-ray on 30-day mortality in the mouse. Quarterly report of Biological and Medical Research Division. Argonne National Laboratory, ANL-5597 (1956). pp. 65-69.

RECOVERY IN NEUTRON-IRRADIATED CHICK EMBRYOS

Howard H. Vogel, Jr.

In previous work[1] it was established that a single exposure of approximately 450-650 rads of fission neutrons was necessary to kill 4-day chick embryos within 6 hr after exposure.

Two experiments have recently been carried out in order to determine whether recovery occurs when such a lethal neutron dose is fractionated into two exposures with a short time between them. It is evident from the data (Table 15) that a single exposure of fission neutrons of 640-650 rads, delivered in 100 min as a single dose to 4-day-old chick embryos *in ovo*, will kill more than 90% within a 6 to 7-hr period after exposure. The criterion for survival was detectible heart beat when the egg was

TABLE 15

Comparison of effects of single and fractionated neutron exposures in 4-day-old chick embryos irradiated *in ovo*

Irradiation	Exposure time, min	Dose, rads	Time of incubation after beginning of irradiation, hr	No. dead / No. exposed	Mortality at 6-7 hr, %
Experiment I					
Single exposure	100	643	6.5	13/15	87
Fractionated exposure*					
First fraction	33	214	7†	1/15	6
Second fraction	67	429			
Unirradiated controls	-	-	-	0/14	0
Experiment II					
Single exposure	100	~650	6.5	17/18	94
Fractionated exposure**					
First fraction	50	325	6†	1/18	5
Second fraction	50	325			
Unirradiated controls	-	-	-	0/8	0

*Fractionated 1/3: 2/3, with 155 min between the two exposures.
**Fractionated 1/2: 1/2, with 120 min between the two exposures.
† Time after beginning of second exposure.

opened and the embryo examined Of the 33 embryos irradiated in the two experiments, 30 were clearly dead when examined, but 3 were classed as alive since their hearts did show some slight, erratic beating It was evident, however, from large hemorrhages and drained extra-embryonic vascular areas, that these 3 embryos could not have lived for more than a few hours. Therefore, for our purpose, this single exposure to fission neutrons (650 rads) can be considered approximately a 6-hr LD_{99}.

When this same dose was delivered in two fractions with a short time interval (120 or 155 min) between the two exposures, there was evidence of dramatic and rapid recovery

In Experiment I (Table 15), the total dose was divided into a first component (1/3) and a second component (2/3) with approximately $2\frac{1}{2}$ hours between exposures. In Experiment II, the dose was equally fractionated with 2 hours between the two exposures. In both experiments, the eggs were kept in an incubator between the two exposures and returned to the incubator after the second exposure. Dose rate was constant for all experiments, with the CP-5 reactor operating at 2000 kw Under these conditions, only 1 embryo (5%) died in each experiment during the 6-hr period of observation Furthermore, there were few signs of intraembryonic hemorrhage in any of these embryos, in contrast to the massive hemorrhages in brain, heart, amnion, and allantois observed in the embryos of the single-exposure group. The embryos receiving the fractionated exposures also exhibited good extra-embryonic circulation in contrast to those of the single exposure group; in the latter, the entire system was usually drained of blood. The embryos receiving the divided dose appeared to be in good condition generally, except for several small petechiae in the extra-embryonic area, especially near the sinus terminalis.

It is evident from the results of this experiment that as short a period as 2 hr between neutron exposures allows a significant degree of recovery from the effects of the first dose. It is not impossible that all of the first dose is recovered in this time since 429 rads in the first experiment is just sublethal and might account for the single death without any contribution from the preliminary exposure of 214 rads.

These experiments are interesting since they establish a recovery time for the chick embryo exposed to fission neutrons of the same order of magnitude as that reported by Stearner and Tyler following Co^{60} γ-rays.[2] Further experiments are planned to try to investigate this rapid recovery phenomenon in the irradiated chick embryo.

References

1. Vogel, H. H., Jr. and D. L. Jordan. The relative biological effectiveness of Co^{60} γ-rays and fission neutrons: Lethal effects on the 4-day chick embryo. Radiation Research 11: 667-683 (November, 1959).

2. Stearner, S. P. and S. A. Tyler. An analysis of acute radiation mortality in the chick. I. Indicators of injury in the chick and chick embryo. Radiation Research 7: 453-454 (1957). (Abstract)

MODIFICATION OF THE MEMBRANE FILTER TECHNIQUE FOR STUDIES OF RADIATION EFFECTS IN SPORES OF BACILLUS MEGATERIUM

B. F. Kaleta and E. L. Powers

The technique of using 47.0-mm Millipore Filters* (MF's) and its application in radiation studies of Bacillus megaterium spores has been reported previously.[1] The desirability of limiting the size of the radiation field for certain experiments led to modifications in the above method that are reported here.

Materials and Methods

The spores of Bacillus megaterium, ATCC #8245, were prepared and mounted on 47.0-mm MF's in the manner previously described with the exception that about 25 times as many spores were mounted for each desired concentration. These were dried in vacuo, and then smaller discs, 6.8 mm in diameter, were punched out with a cork borer. They represent approximately 1/25 of the area of the larger disc and therefore carry approximately 1/25 of the original number of spores. For germination, a plating technique involving two MF layers instead of the usual one was devised. Prior to incubation at 35°C, 0.5 ml of a buffered peptone-glucose medium (double-strength Difco MR-VP Medium) was pipetted onto a 27.0-mm absorbent pad, the pad was covered with a 27.0-mm MF and five 6.8-mm MF's were placed on this. To have a statistically useful number of survivors on these small MF's, it was necessary to crowd them with numbers of spores that would result in confluence if incubated for the usual 16 hr. Therefore the incubation period was reduced from 16 to 7 hr. Table 16 shows two experiments designed to validate the use of the double MF plating technique and the reduction of the time of incubation to 7 hr. The number of colonies was the same whether one or two MF's was used on the pad or whether the colonies were incubated for 7 hr or for 16 hr. Before 7 hr some of the colonies are barely visible and after that time, confluence begins to occur. Even at 7 hr the colonies are quite small, the average size being about 0.2 mm. To aid in counting, the MF's and pads were placed in a sealed chamber with a dish containing a few drops of a 2% aqueous solution of osmium tetroxide. After from 7 to 16 hr of exposure to the vapors, the colonies appear dark brown, shiny and raised above the surface of the dull, lighter brown MF.

An experiment was done to determine the maximum number of spores that could be mounted on the 6.8-mm MF's and still show 100% colony formation. The linear character of the zero intercept curve (Figure 24) that relates relative spore count to colony-count shows that the colonies from

*Millipore Filter Corporation, Watertown, Mass.

TABLE 16

Comparison of plating efficiencies of single and double plating techniques, and 16 hr and 7 hr incubation times

Incubation time, hr	Method of plating	No. of colonies		Recovery, %
		Expected	Observed	
16	27.0 mm MF on 27.0 mm Pad	-	153	100.0
7	"	765	740	96.7
7	27.0 mm MF on 27.0 mm MF on 27.0 mm Pad	765	757	99.0
16	47.0 mm MF on 47.0 mm Pad	-	56	100.0
7	"	280	276	98.6
7.5	"	280	271	96.8

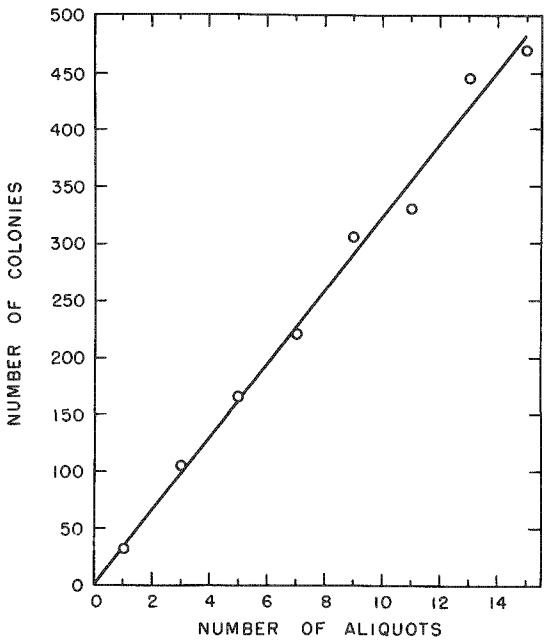

Figure 24

Relationship of the aliquot number to the number of microcolonies counted after 7 hr of incubation at 35°C. Semimicro method, using 6.8-mm MF's.

as many as 445 spores grown in that small area for 7 hr can be accurately counted with no evidence of loss in plating efficiency up to that point.

The X-ray source was a Machlett OEG 60-tube with a tungsten target, beryllium window, no added filtration (HVL 0.070 mmAl), operated at 50 kv and 45 ma. For the irradiations, the 47.0-mm MF's were piled in stacks of five, whereas, five of the 6.8-mm MF's were singly placed within the area occupied by the larger MF's. The target distance was 15 cm, and the dose rate to the first filter was 19,860 r/min. All of the irradiations were done in air at room temperature.

Results

An inactivation curve obtained by the 6.8-mm MF method with 7-hr incubation (semimicro method) is compared to one obtained by the usual 47.0-mm MF method with 16-hr incubation (macromethod) as shown in Figure 25. The points represent the average fraction surviving on five MF's. In the case of the 47.0-mm MF's, the dose represents the average through the stack. No significant difference is seen in the results when the two methods are compared.

It can also be seen in Figure 25 that the initial spore concentration on the MF apparently does not affect the fraction surviving X-rays within the limits tested. MF's were mounted with a fixed spore concentration,

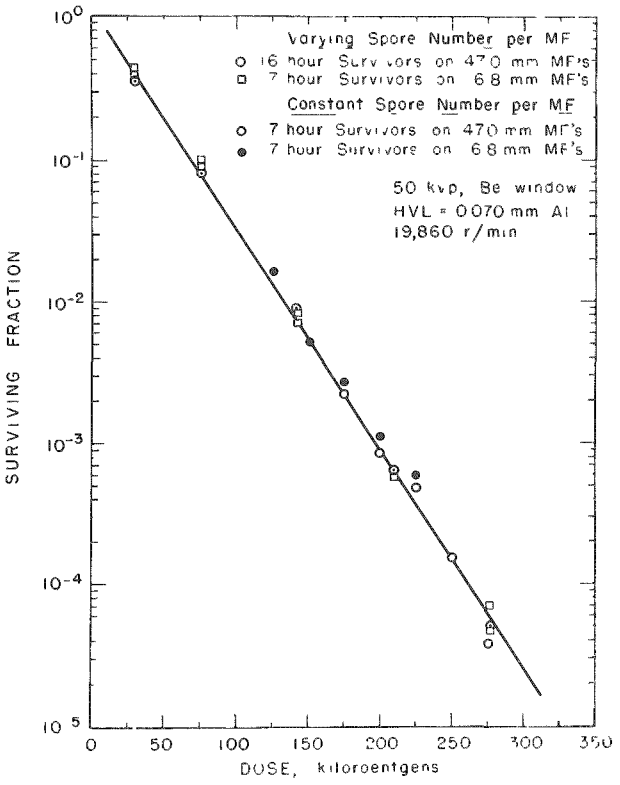

Figure 25

Survival curve for B. megaterium spores. The character of the curve is independent of incubation time, spore concentration, and MF size within the limits tested. The constant spore number represents about 5×10^5 spores per MF for every dose. In varying the spore number per MF, the concentration of spores was increased 10 times for each order of magnitude of inactivation.

5×10^5 per MF, and then given a range of X-ray doses, the range being limited by the number of survivors at the higher and lower doses. Both 47.0-mm MF's and 6.8-mm MF's were used as indicated in the figure.

The semimicro method is a practical technique by which X-ray inactivation curves can be obtained for work in which the size of the radiation field is limited. In addition to this there is an increase in efficiency over the macro method. This is because ten 6.8-mm MF's can be punched from one 47.0-mm MF, and 10 times as many data can be obtained with no increase in the work of preparation. Furthermore, less medium is used for the growth of colonies, and the incubation period is shorter. When the radiation field size is not limited, 5 or more 6.8-mm MF's can be inactivated in an isodose field equivalent to that occupied by one large MF.

Reference

1. Powers, E. L., C. F. Ehret, and A. Bannon. The membrane filter technique in radiation studies of spores of Bacillus megaterium. Appl. Microbiol. 5: 61-64 (1957)

STRONTIUM-85 RETENTION BY THE RAT AS A FUNCTION OF AGE AT INJECTION

Thomas W. Speckman and William P. Norris

The dependence of retention of a bone-seeking isotope upon the age of an animal at the time of injection has been noted in previous reports.[1] The following analysis represents an effort to express in generalized mathematical terms the results of several such experiments to determine retention of Sr^{85} in rats. Such treatment makes the results more useful in correlations with the results of studies of other isotopes and other species.

In all, 68 rats were injected intravenously with Sr^{85} at ages varying from 4 to 428 days. Retention in these animals was measured periodically using an external γ-counting method, which allowed sequential observations of the same animals for the entire experimental period. For presentation here the animals were somewhat arbitrarily combined into six groups having the following ranges of age at injection: 4-30, 42-61, 82-141, 162-233, 252-338, 366-428 days.

The retention data for each group, excluding the first few days following injection, may be described by the sum of two exponential terms. Each of these exponentials seems to retain the same slope regardless of age of the animals at injection. The shorter-term exponential has a slope of approximately -0.041, representing a half-life of 16.9 days, while the long-term component has a slope in the order of -0.0007, representing a half-life of 990 days. However, the estimation of the latter value has such a large associated error that it provides only an approximation of the true value. Unfortunately, it is not possible to establish the existence of a finite number of exponential terms by inspection of a curve of the sort found in these experiments -- regardless of the quality of the data obtained. The representation described above represents the most naive and simplified solution to the mechanistic problems involved. However, this approach illustrates that the shape of the terminal portions of the age-dependent retention curves are very similar. Indeed, since they cannot be shown by statistical methods to be dissimilar, they are presumed to be identical.

The modified power function described by Norris et al.[2] and Tyler[3] affords an over-all empirical description of the data in a simple mathematical form which can be readily integrated and differentiated. The modification is made by introducing γ, a factor which forces the power function to conform to the physical requirement that retention must be unity when $t = 0$. The modified form representing retention following a single injection, where the physical half-life of the isotope is not taken into account, is

$$R_t = \frac{(t+\gamma)^b}{\gamma^b} \cdot \quad t \geq 0, \; -1 < b < 0, \; \gamma > 0 \qquad (1)$$

where R = fraction of injected dose retained, t = days since injection, and b = slope of the power function described by Equation 2. This form approaches the power function

$$R_t = At^b \cdot t \geq 1 \qquad (2)$$

(where A = intercept with the retention axis at one day) asymptotically as t increases.

When the retention data from the old rats are plotted on log-log paper, they closely approximate a straight line. Thus when one uses a straight-line power function to represent retention in the oldest groups, 366-428 days at injection, a least squares linear regression analysis results in b = -0.35 ± 0.02. Since, as stated earlier, the shapes of the terminal portions of the retention curves are considered to be identical in all age groups, b becomes a property of the power function asymptote which rats of all ages will ultimately approach. The quantity γ now becomes the sole age-dependent factor in Equation 1. With Equation 1 used to describe retention in rats of all ages at injection, γ was found for each experimental age group by a least squares linear regression of the relation.

$$R_t^{\frac{1}{b}} = 1 + \frac{t}{\gamma} \quad . \qquad (3)$$

These results are shown in Figure 26 with a smooth curve through the points. By use of Equation 1 the calculated retention resulting from injection at specific ages results in the curves shown in Figure 27. Appropriate values for γ were taken from the smooth curve in Figure 26.

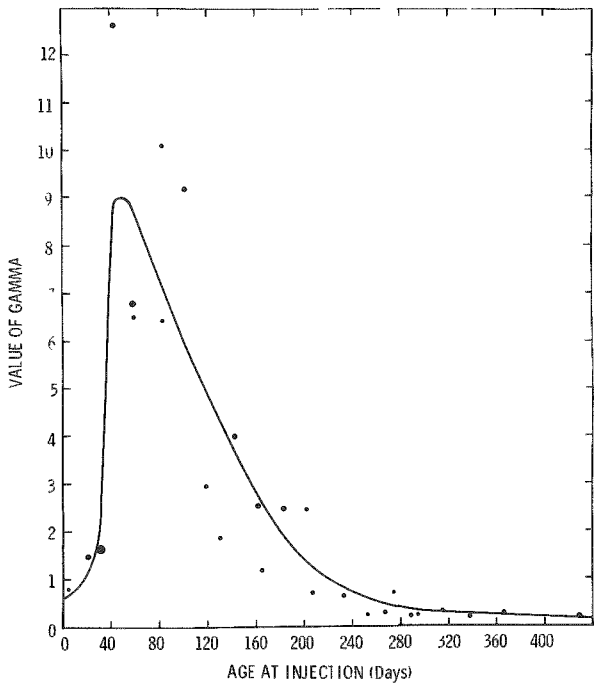

Figure 26. Variation of γ as a function of age at injection.

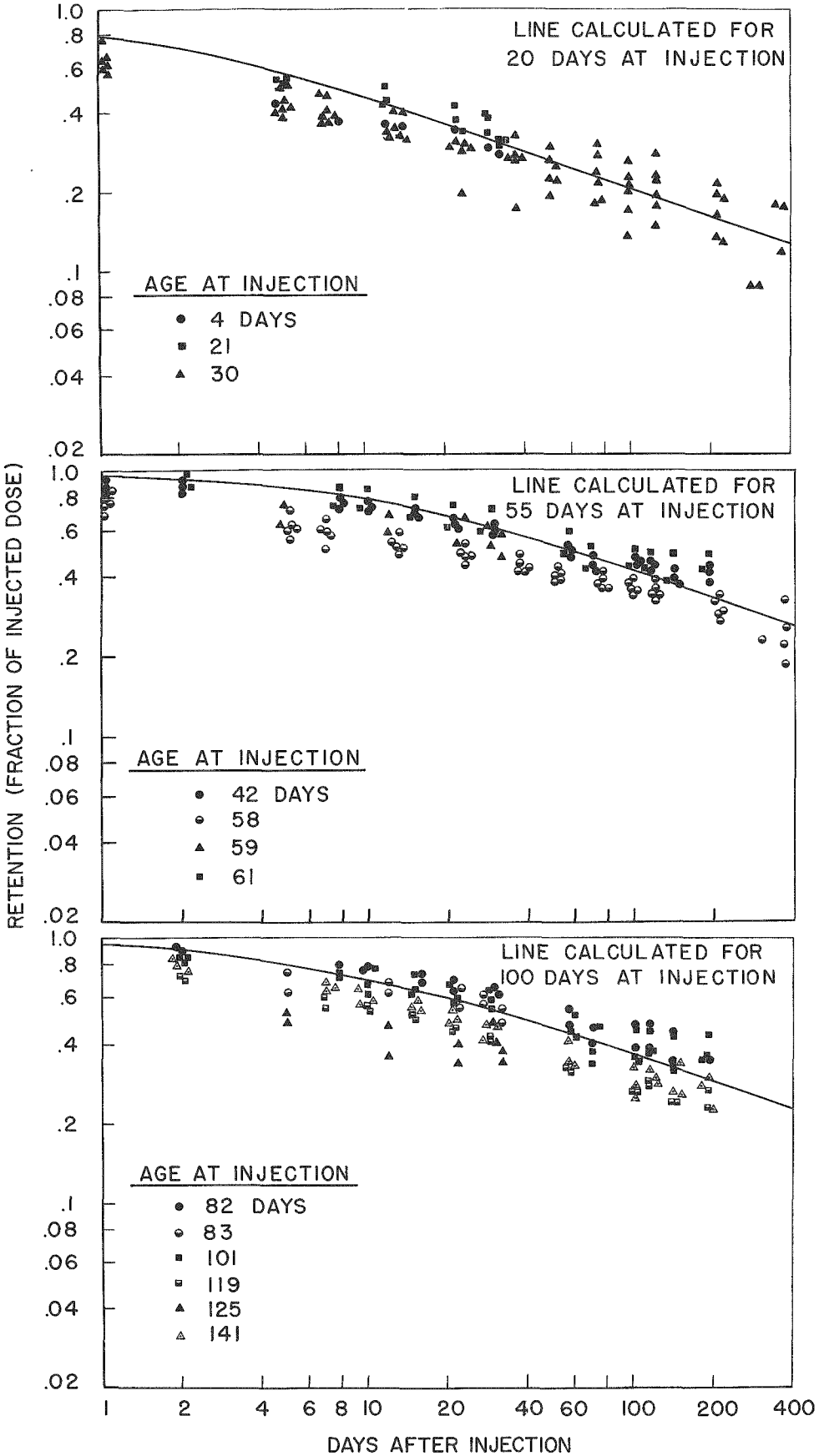

Figure 27. Retention of Sr^{85} in rats.

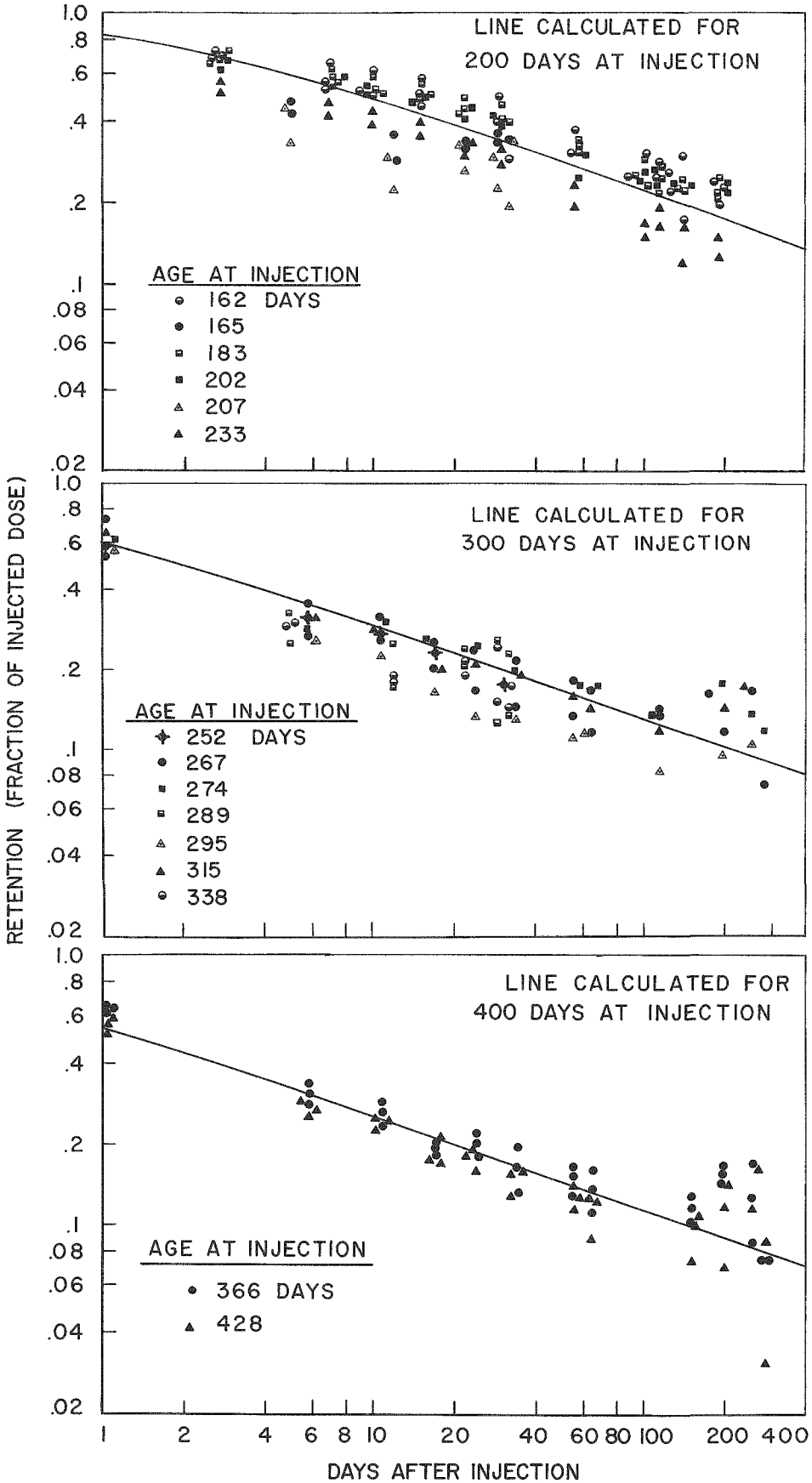

Figure 27. Retention of Sr^{85} in rats.

The experimental retention measurements, corrected for natural decay of Sr^{85}, are plotted on the same graph as the calculated curve representing the closest age at injection. It must be pointed out that within each age range, the line plotted is calculated for the specific age noted; hence, for each specific age group represented by data, the points representing its retention curve should appear in proper relation in a sequential family of curves covering the age span represented. The experimental points are seen to fit into such a pattern reasonably well.

Figure 28 is a composite of Figure 27 without the experimental data points. The power function asymptotes are shown as broken lines to indicate the variance from the straight power function introduced by the modification invoking the use of γ. This illustration points out the importance of using the modified form of the power function unless concerned only with adult animals.

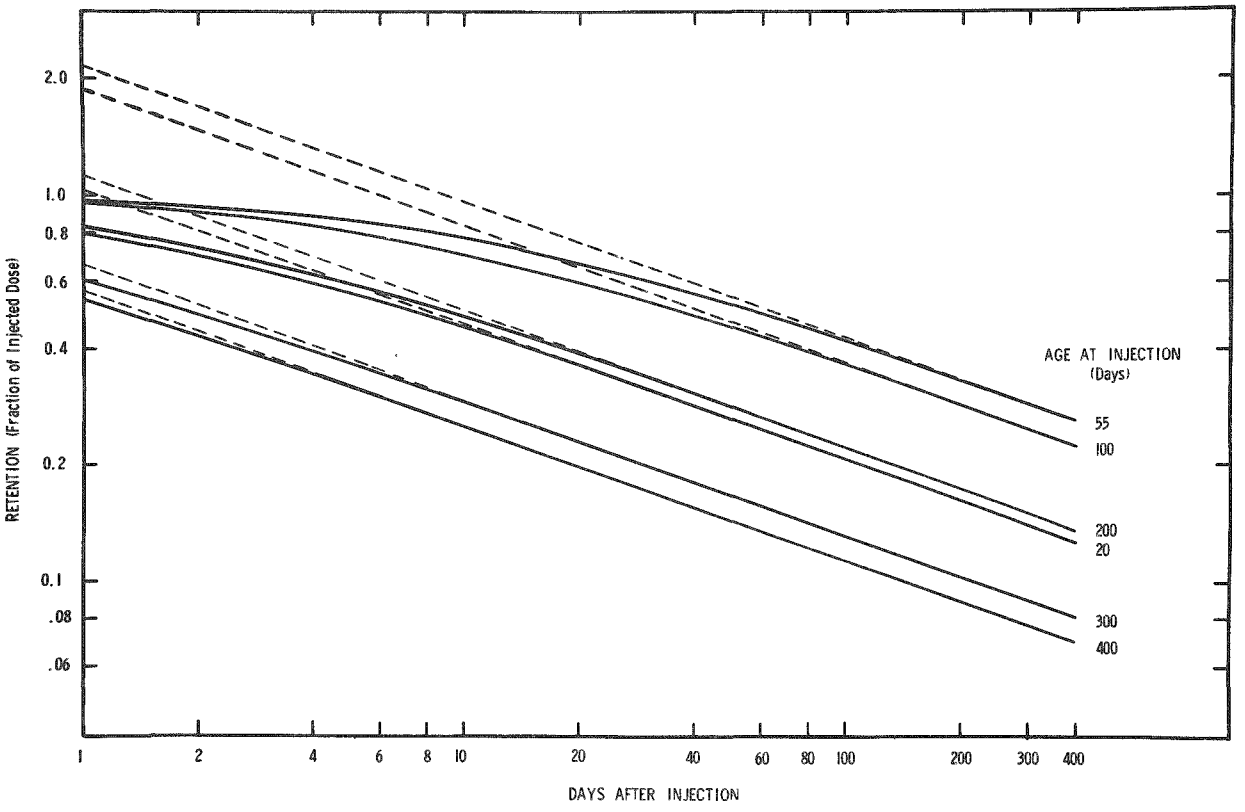

Figure 28. Retention of Sr^{85} in rats as influenced by age at injection.

The material in this report is being analyzed more thoroughly and will appear shortly as a more extensive publication.

We gratefully acknowledge the assistance of Sylvanus A. Tyler in the mathematical portion of the work, and the technical assistance of Mrs. C. Armstrong.

References

1. Speckman, T. W., and W. P. Norris. The retention of strontium^{85} in rats as a function of animal age at injection. Quarterly Report of Biological and Medical Research Division. Argonne National Laboratory, ANL-5597 (1956) pp.77-78.

2. Norris, W. P., S. A. Tyler, and A. M. Brues. Retention of radioactive bone-seekers. Science 128: 456-462 (1958).

3. Tyler, S. A. On a modification of the power function. Biological and Medical Research Division Semiannual Report. Argonne National Laboratory, ANL-5841 (1957) pp. 132-138.

PROGRESS REPORT: GRANULOCYTE LIFE CYCLE. III.

Mary A. Maloney and Harvey M. Patt

In this communication we wish to present data pertaining to the kinetics of granulocyte production in the normal dog. This study is based on high resolution radioautography after administration of tritium-labeled thymidine. Experimental procedures were described previously.[1,2]

Tritiated thymidine is available for incorporation into DNA only for a very brief period after injection since it is rapidly metabolized and excreted.[3] The label appears first in myeloblasts, promyelocytes, and myelocytes, cells that ordinarily contribute to renewal of the granulocyte population by mitosis. Since, on the average, half of the label will be transferred to each daughter cell, labeled cells should accumulate in marrow until there is a significant dilution through subsequent mitoses or maturation and release to peripheral blood. In point of fact, the number of labeled cells, computed in terms of a normalized distribution of the various cell types, increases during the first 36 to 48 hr after intravenous injection of tritiated thymidine in a dosage of 0.1 to 0.3 $\mu c/g$. The mean generation time of the proliferating cells can be estimated from the rate of increase and is of the order of 15 hr.

A more detailed picture of the generation cycle may be obtained from the temporal distribution of labeled mitotic figures following injection of tritiated thymidine. A correlation of this sort is being made, and although the study has not been completed, the approximate sequence of events may be discerned from preliminary analyses. It has been observed that mitotic figures for myeloblasts, promyelocytes, and myelocytes are labeled within 30 to 60 min after thymidine administration. There is a maximum at 4 to 5 hr with a sharp decline at 6 hr, and a second increment of labeled mitoses at 14 to 15 hr. The peaks in labeled mitoses are paralleled by an increase in the number of labeled myeloid cells. A mean generation time of 13 to 14 hr may be inferred from these data. It would appear that mitosis begins within an hour after completion of DNA synthesis and that the duration of mitosis is about an hour. The interval between completion of mitosis and onset of DNA synthesis is approximately 7 to 8 hr and the DNA synthetic period is about 4 to 5 hr.

References

1. Maloney, M. A., and H. M. Patt. Progress Report: Granulocyte life cycle. Biological and Medical Research Division Semiannual Report. Argonne National Laboratory; ANL-5841 (1958) pp. 144-146.

2. Maloney, M. A., and H. M. Patt. Neutrophil life cycle with tritiated thymidine. Proc. Soc. Exptl. Biol. Med. 98. 801-803 (1958).

3. Hughes, W. L., V. P. Bond, G. Brecher, E. P. Cronkite, R. B. Painter, H. Quastler, and F. G. Sherman. Cellular proliferation in the mouse as revealed by autoradiography with tritiated thymidine. Proc Natl. Acad. Sci. U. S. 44 476-483 (1958).

PROGRESS REPORT: PYRESIS STUDIES

V. Sedimentation Rate Studies

Marcia White Rosenthal and Joan F. Fried

A significant increase in sedimentation rate in rats follows the artificial production of fever by the subcutaneous injection of yeast. It has been shown that injection of an antipyretic dose (125 mg/kg) of aurintricarboxylic acid (ATA), but not of sodium salicylate, prevents this increase.(1)

The present report summarizes our further work with these substances in sedimentation rate studies: the determination of dose effects in vivo and a comparison of the effectiveness of the two substances in vitro when added to either rapidly sedimenting rat or human blood.

In vivo experiments. The production of fever in rats, the accompanying increase in sedimentation rate, and the procedures used in the studies of sedimentation rate were the same as described earlier.(1) Intravenous injection of 75 mg/kg of ATA, an effective antipyretic dose, was as effective as the previously used dose of 125 mg/kg in preventing the increased sedimentation rate associated with the yeast-induced fever, while a dose of 35 mg/kg, ineffective as an antipyretic, was only partially effective in the sedimentation rate test. Blood samples were taken 1 hr after the injection of ATA. In a second series of experiments, summarized in Table 17, blood was taken at increasing intervals after intravenous administration of 125 mg/kg of ATA. ATA exerts a significant but decreasing effect on the sedimentation rate after it has been in the blood for 6 hr and has lost its effect after 9 hr. Sterilized ATA was found to be fully effective as an antipyretic and in preventing the increased sedimentation rate: a 6% solution of the ammonium salt was autoclaved at 15 pounds pressure for 20 min, then injected intravenously at a dose of 125 mg/kg.

We have found it impossible to decrease the sedimentation rate in vivo with sodium salicylate, either by single intraperitoneal injections of up to 500 mg/kg or by artificially maintaining a high blood salicylate level through ligation of the renal blood vessels. In the latter experiments the kidneys were ligated bilaterally with the rat under ether anesthesia about 18 hr after administration of yeast. The rats were injected intravenously 3 to 5-$\frac{1}{2}$ hr later either with saline or with 250 mg/kg of sodium salicylate, the largest dose which is nontoxic under these conditions. Either 1 or 4 hr after this injection, blood was withdrawn for determination of sedimentation rate. Even after the

TABLE 17

Effect of ATA *in vivo* on sedimentation of red blood cells in rats

Treatment*	Hr after treatment	No. rats	RBC sedimentation**		Estimated blood content ATA,† mg/ml
			Mean, mm	S.E.	
Saline	1	13	17.5	2.6	----
Saline	4	7	17.1	2.6	----
Saline	9	7	19.5	4.6	----
ATA	1	8	Trace	---	0.43
ATA	2	5	Trace	---	0.35
ATA	4	6	0.9	0.4	0.27
ATA	6	5	2.0	0.7	~0.22
ATA	9	7	12.4	3.0	~0.15
ATA	12	6	24.9	3.7	0.14

*125 mg/kg of ATA, or an equivalent volume of saline, was injected intravenously about 18 hr after subcutaneous administration of 3.0 ml of a 15% yeast suspension in saline.

**Determined 2-$\frac{1}{2}$ hr after sampling.

†Calculation based on measured disappearance of ATA from blood following intravenous injection of ~77 mg/kg (19.2 mg) NH_4ATA,[2] assuming that the blood retention is the same following 125 mg/kg. Figures for 5 and 9 hr are estimated from the curve since actual readings were not made at these intervals.

4-hr interval, the sedimentation rate of the treated rats was not significantly altered from that of the saline controls. For example, 2-$\frac{3}{4}$ hr after blood was drawn, the mean fall of the red blood cells in the treated group (7 rats) was 32.8 mm $\pm$ a standard error of 8.45, as compared to 40.6 $\pm$ 3.4 mm in the controls (5 rats). Rough quantitative measurements of the salicylate in the pooled blood of these rats using a colorimetric method based on development of a complex with ferric nitrate,[3] indicated that salicylate had indeed been maintained at the level present immediately after injection (~5 mg/ml of blood). This failure of sodium salicylate to affect the sedimentation rate is surprising in view of its effect in rheumatic fever and in the *in vitro* experiments to be described.

In vitro experiments. These were performed in a manner similar to that used by Formijne.[4] Rapidly sedimenting rat blood was removed by cardiac puncture into a heparinized syringe about 20 hr after subcutaneous administration of 3 ml of a 15% yeast suspension. One-ml aliquots of blood were immediately added to test tubes containing 0.2 ml of a test solution or of 0.85% saline. After mixing, the blood was placed in Wintrobe tubes, and the sedimentation of the red cells was observed frequently for at least 2-$\frac{1}{2}$ hr, and at 22 hr. Table 18 summarizes the results at 2-$\frac{1}{2}$ hr, when the sedimentation of the red blood cells had essentially ceased. Readings at 22 hr were somewhat lower, but the relationships between groups were unchanged. Under the conditions of these experiments, sodium salicylate had the expected effect upon the sedimentation rate. ATA was somewhat more effective. A comparison of the last columns in Tables 17 and 18 shows that for maximum effect about four times as much ATA was required in vitro as in vivo.

TABLE 18

Effects of ATA and salicylate in vitro on sedimentation rate of red blood cells of rats

Test solution		No. rats	RBC sedimentation*		Concentration of test solution in tube, mg/ml
Compound	Concentration, %		Mean, mm	S.E.	
Saline	0.85	18	15.0	1.3	----
Na Salicylate	0.5	6	6.8	4.0	0.83
Na Salicylate	1.0	6	0.5	0.3	1.66
Na Salicylate	2.0	6	0.1	0	3.33
ATA	0.25	6	15.9	5.8	0.42
ATA	0.5	6	1.7	0.3	0.83
ATA	1.0	6	0.2	0.02	1.66

*Determined 2-$\frac{1}{2}$ hr after sampling. Tubes contained 1 ml blood and 0.2 ml test solution.

This in vitro test was performed also with freshly drawn, rapidly sedimenting human blood from rheumatic fever patients.* Aliquots of blood from each of four patients were tested as above with 0.5, 1.0 and 2.0% solutions of both sodium salicylate and NH_4ATA, and with a

*We are indebted to Doctors Albert Dorfman and Burton J. Grossman and to Miss Dorothy Batterton of the Thomason Laboratory of La Rabida Sanitarium for this opportunity and for drawing the blood samples.

saline control. Only the 2.0% solutions slowed the sedimentation rate significantly in comparison to that of the control: in two cases to a normal rate, and in two cases with abnormally low hematocrits and very rapid initial sedimentation rates, to an intermediate rate. ATA and sodium salicylate were roughly equivalent

The failure of injected salicylate to change the sedimentation rate in the rat under the conditions of our experiments becomes more interesting in view of this demonstration that in vitro it is capable of returning sedimentation rate to normal Further, our experience with ATA, and the clinical comparisons of salicylate under in vivo and in vitro conditions, indicate that the in vitro condition usually requires more of the test substance. The failure of salicylate may be attributable to insufficiently high blood levels, although this explanation becomes less credible in view of the results of our experiments in which the kidneys were ligated. Some factor present in the whole rat, and not in the isolated blood, evidently prevents sodium salicylate but not ATA from exerting an effect upon the sedimentation rate under the conditions of our experiments.

References

1. Rosenthal, M. W., and J. F Fried. Progress Report: Pyresis Studies. IV. Hematocrit and sedimentation rate during antipyresis. Biological and Medical Research Division Semiannual Report. Argonne National Laboratory, ANL-5841 (1958) pp. 149-152

2. Schubert, J , M. R. White and A Lindenbaum. Studies on the mechanism of protection by aurintricarboxylic acid in beryllium poisoning. I. J. Biol. Chem. 196 279-288 (1952)

3. Gradwohl, R. B. H Clinical Laboratory Methods and Diagnosis. 5th Ed., Vol. II, St. Louis. The C. V. Mosby Co., 1950.

4. Formijne, P. Effects of benzoic acid and hydroxybenzoic acids on the sedimentation rate of red blood corpuscles. Koninkl. Nederl. Akademie Van Wetenschappen - Amsterdam. Proc., Series C, 58 (3) 328-335 (1955).

PROGRESS REPORT: LEAD REMOVAL

I. Effect of Three Polyamino Acid Chelating Agents on Acute Experimental Lead Poisoning in Rats

Joan F. Fried, Jack Schubert and Elizabeth Moretti

The value of ethylenediaminetetraacetic acid (EDTA) in the clinical treatment of chronic lead poisoning in humans is now fairly well established.[1] Recently two compounds which are structurally related to EDTA have been shown to be more effective than EDTA in the removal of radioelements from laboratory animals.[2,3,4] These are diethylenetriaminepentaacetic acid (DTPA) and 2:2'-bis (dicarboxymethyl) aminodiethylether (BAETA). For these reasons, it was of interest to compare the action of these two agents with that of EDTA in the therapy of acute experimental lead poisoning in rats.

Two types of experiments were performed. In one, CaEDTA, CaDTPA, and saline were compared with respect to their effects on the survival of groups of rats given a lethal dose of lead nitrate and treated one hour later. In the other, the toxicity of the lead chelates of BAETA, DTPA and EDTA was determined after their administration as single, intraperitoneal injections into rats.

Methods

Lead nitrate, $Pb(NO_3)_2$, prepared by solution in distilled water without adjustment of pH, was injected intravenously into rats at a dose of 70 mg/kg as Pb. CaEDTA and CaDTPA were prepared by neutralization of the acids with NaOH, addition of $CaCl_2$ in 1:1 molar ratio of Ca to chelating agent, and adjustment of the pH to 7.2. These agents were given intravenously in doses of 118 mg/kg and 158 mg/kg respectively (equimolar ratio). PbBAETA, PbDTPA and PbEDTA were prepared by neutralization of the acid forms of the chelating agents with NaOH, addition of $Pb(NO_3)_2$ in 1:1 M ratio of Pb to chelating agent, and adjustment of the pH to 7.0. They were given intraperitoneally in doses ranging from 110 to 1260 mg/kg as Pb. Sprague-Dawley female rats of 3-6 months of age and weighing 225-285 g were used; animals of equal weight and age were selected for each experiment.

Results

The results of the survival experiment are shown in Table 19. There was no significant difference in survival among the three groups. However, the prolonged time to death in treated groups as compared with the saline controls may have some significance, especially in the case of the CaEDTA-treated group.

TABLE 19

Effect of equimolar levels of CaEDTA (118 mg/kg) and CaDTPA (158 mg/kg) given intravenously 1 hr after a lethal dose of $Pb(NO_3)_2$*

Treatment	No. of animals	Survival, %	Avg. time to death of nonsurvivors, days
CaEDTA	10	40	2.2
CaDTPA	11	36	1.4
Saline (controls)	12	33	0.9

*$Pb(NO_3)_2$, 70 mg/kg as Pb, intravenously.

The toxicities of the lead chelates of EDTA, DTPA and BAETA are given in Table 20. PbDTPA is somewhat more toxic than PbEDTA, but both are considerably less toxic than unchelated Pb^{++} ion. On the other hand, PbBAETA had almost the same order of toxicity as unchelated Pb^{++}. Our preliminary studies on the toxicity of CaBAETA indicate that this cannot be attributed to any toxic effects of the BAETA component.

TABLE 20

Toxicity of PbEDTA, PbDTPA and PbBAETA given intraperitoneally to rats

Dose of Pb, mg/kg	Survival, %		
	PbEDTA	PbDTPA	PbBAETA
110	------	------	37 (8)
140	------	------	0 (4)
175	------	------	0 (4)
945	100 (3)	100 (4)	-----
1260	100 (7)	50 (8)	-----

Numbers of animals injected are in parentheses.

Discussion

The lack of therapeutic effect of both EDTA and DTPA in acute lead poisoning is in contrast to the effect of both of these compounds in acute manganese poisoning.[5] The stability constant of EDTA for Pb^{++} is more favorable than for Mn^{++}; K_f PbEDTA = 18 and K_f MnEDTA = 14.[6] However, the affinity of Pb^{++} for the tissue proteins is much greater than that of Mn^{++}. At 1 hr after intravenous injection of Pb^{++}, about 30% of the injected dose is in the liver and kidneys and about 20% in the plasma.[7] Hence, the net stability constants of EDTA and DTPA for Pb are too low; these compounds cannot compete with the proteins of these tissues sufficiently to effect removal of a significant fraction of their lead content.

When lead is injected in the chelated form, however, the chelates of EDTA and DTPA are sufficiently stable to permit the administration of a relatively large amount without toxic effects. While no data are available for the stability constant of BAETA with Pb^{++}, it is apparent from our results that it must be orders of magnitude lower. This is probably due to steric hindrance.

References

1. Chenoweth, M. B. Chelation as a mechanism of biological action. Pharmacol. Rev. 8 77 (1956).

2. Catsch, A., D. K. Lê, and H. Melchinger. Untersuchungen über therapeutische Möglichkeiten bei Vergiftungen mit radioaktiven spaltprodukten. Strahlentherapie 106 606-625 (1958).

3. Fried, J. F., E. H. Graul, J. Schubert, and W. M. Westfall. Superior chelating agents for the treatment of plutonium poisoning. Atompraxis 5 (1) 1-5 (1959).

4. Smith, V. H. Removal of internally deposited plutonium. Nature, 181 1792 (1958).

5. Fried, J. F., J. Schubert, and E. Moretti. Progress Report: The use of chelating agents in the treatment of manganese poisoning. III. Biological and Medical Research Division Semiannual Report. Argonne National Laboratory, ANL-5841 (1957) pp. 155-157.

6. Bjerrum, J., G. Schwarzenbach, and L. G. Sillen. Stability Constants Part 1: Organic Ligands, London: The Chemical Society, 1957 p. 77

7. Schubert, J., and M. R. White. Effect of sodium and zirconium citrates on distribution and excretion of injected radiolead. J. Lab. Clin. Med. 39 260-266 (1952).

PROGRESS REPORT: TREATMENT OF RADIOSTRONTIUM POISONING. II.

Arthur Lindenbaum, Joan F. Fried and Elizabeth S. Moretti

A previous report has dealt with the promotion of increased radiostrontium excretion from the body by means of the salts of rhodizonic acid.(1) The present report extends this study and describes work aimed at determining the effect of a diuretic, Diamox® (acetazolamid), used in conjunction with sodium rhodizonate (NaR)* on rats given Sr^{85}.

The rats received 0.8 μc carrier-free $S^{85}Cl_2$ in 1 ml of water given orally by stomach tube, followed immediately, using the same tube, by 7 ml of either water, Diamox (7 mg/kg), NaR (90 mg/kg), or NaR plus Diamox (90 mg/kg and 7 mg/kg respectively). At 1-½ hr after administration of Sr^{85}, the original therapeutic or control treatment was repeated. The animals were sacrificed 24 hr after administration of Sr^{85}. Carcass, femurs, urine and feces were assayed for Sr^{85} by means of a scintillation counter. Some typical results are shown in Table 21.

TABLE 21

Effect of sodium rhodizonate (NaR) and Diamox on the distribution of orally administered Sr^{85} in rats.

No. animals	Treatment	Percent of Administered Sr^{85}			
		Carcass less GI tract, mean ± σ*	Skeleton,** mean ± σ	Urine, mean ± σ	Feces plus GI tract, mean ± σ
5	Diamox only	36.44 ± 3.33	24.48 ± 1.35	2.65 ± 0.04	62.98 ± 1.98
5	NaR + Diamox	24.15 ± 2.98	14.19 ± 1.42	8.02 ± 1.14	66.53 ± 3.30
5	Water only	27.02 ± 3.71	17.47 ± 2.33	2.29 ± 0.31	69.81 ± 3.67
5	NaR only	27.93 ± 4.39	11.82 ± 0.75	5.95 ± 0.37	64.33 ± 3.34

*σ = std. error of the mean.

**Arbitrarily calculated as activity of both femurs times 10.

It may be noted that in comparison to either of the control groups (water only, or Diamox only) the groups given NaR show a significantly lower burden of skeletal Sr^{85} and a corresponding elevation of the urinary Sr^{85} ($P < 0.05$, or better). The differences in skeletal content amounted to over 40% in the groups given Diamox and over 30% in groups receiving

*The sodium and potassium salts appear to be therapeutically equivalent.

no Diamox. It appears that these differences do not represent a true reduction of skeletally deposited Sr^{85} but rather the shunting of circulating Sr^{85} into the urine before skeletal deposition occurs.

Curiously, comparison of the groups receiving either water alone or Diamox alone shows that Diamox induces a significant ($0.01 > P > 0.001$) increase in skeletal Sr^{85}. This effect is produced despite the fact that urinary volume is definitely increased by administration of Diamox.

The increased skeletal retention of Sr^{85} produced by a diuretic agent is an unexpected effect and indicates the need for further work toward an understanding of the mode of action of Diamox, as well as other types of diuretic agents, in connection with radioelement removal.

Reference

1. Lindenbaum, A., M. W. Rosenthal, and J. F Fried. Progress report: Radiostrontium removal. I. Biological and Medical Research Division Semiannual Report, Argonne National Laboratory, ANL-5732 (1957) p. 134.

A NEW PREPARATION OF THE TOXOHORMONE OF TUMORS

Robert N. Feinstein

It is well established that the presence of a tumor will reduce the catalase activity of the liver of the host[1] and, in fact, of most solid tissues.[2] The catalase of the erythrocyte of the host is not affected. It is now generally accepted that the catalase-reducing properties of the tumors lie in a chemical agent elaborated by the tumor and designated "toxohormone" by Nakahara and Fukuoka.[3] Injection of a toxohormone preparation into a normal mouse will reduce the liver catalase of the recipient. Although the literature regarding this agent is now too voluminous to detail in this report, it may be stated that the toxohormone is water-soluble, heat-stable, and insoluble in acetone, ethanol, or ether. Its preparation is based on various combinations of these properties.

The most potent preparations reported are those of Kuzin et al.[4,5] and of Ono et al.[6,7] The first group[5] reports the preparation of chloroplatinate crystals of the active material and the observation of in vivo catalase reductions of 70-80%. This is a greater effect than generally seen, but the authors fail to state the amount of active agent needed to produce such an effect. The Japanese group[6] prepared acetone-dried powder of their tumor, extracted this with a methanol-acetic acid mixture, and precipitated the active agent with ether. The resulting dried powders were active as toxohormone in 20-40 mg doses. By ethanol fractionation, they obtained fractions active at doses of 10 mg. In a later paper,[7] they report further purification to the point where as little as 0.1 mg is demonstrably active.

A technique has been devised which gives active toxohormone fractions with a minimum of time and manipulation. Before describing the method, it should be pointed out that the yield of toxohormone will depend upon the particular tumor used. For example, considerable work was done on preparations from the Walker rat tumor with indifferent results. Active fractions were obtained, but they represented relatively enormous amounts of initial tumor source. For example, the total yield from 20 to 40 g of the tumor, when injected into a 15-g mouse, might cause a reduction in liver catalase of 20 to 40%. This is apparently not due to the particular method employed because Greenfield and Meister,[8] using a quite different technique, also stated that the Walker tumor is poor in toxohormone. We are presently using the Murphy-Sturm lymphosarcoma of the rat and have obtained toxohormone preparations which, injected at a dose of 7 mg of dry powder equivalent to 2 g of original tumor, cause a 30% reduction in liver catalase of the recipient mouse.

The preparative method is as follows. Tumors are excised when they reach perhaps 4 to 10% of total body weight. They are blended in the Waring blender with 5 volumes of distilled water; this total tumor suspension is heated in a boiling water bath for 20 min, then cooled. It has been found that if this material is now frozen overnight, then thawed out the next morning, centrifugation is quick and effective. The precipitate is discarded. To the supernatant, 4 0 M $MnCl_2$ is added, 2.8 ml per 100 ml of supernatant. The $MnCl_2$ is added slowly with constant stirring. The solution is allowed to remain in the refrigerator until precipitation is complete, usually a matter of an hour or two; if necessary, it is kept in the cold overnight.

The Mn-toxohormone precipitate is centrifuged, and the supernatant is discarded. The precipitate is suspended in a small volume of water and homogenized, if necessary, to break up particles; the pH is then raised to 10.0 with NaOH, thus precipitating the manganese as a mixture of its various oxides and hydroxides. After stirring at room temperature for 15 min to assure completion of the reaction, the mixture is centrifuged, and the precipitate is discarded. The supernatant is returned to pH 7 with HCl, and five volumes of acetone are added slowly and with stirring. The mixture is chilled overnight and centrifuged the next morning. The supernatant is discarded, and the precipitate is washed twice with acetone and once with ether, then dried *in vacuo* at room temperature. This is the product which is injected intraperitoneally into weanling mice (15 to 20 g). The liver catalase is assayed 48 hr later because the findings of Greenfield and Meister[8] indicate that the greatest effect is observed at this time.

The method thus has the advantage of requiring no prolonged refluxing no reduction of large volumes of solution and no large volumes of organic solvents.

It has certain disadvantages, however. These are:

1. The preparations tend to be toxic. This may be a manganese toxicity. The LD_{50} for $MnCl_2 \cdot 4\ H_2O$ is approximately 2 mg for a 15-g mouse. Since the exact chemical nature of the mixed oxides and hydroxides of manganese is uncertain, it is not possible to state what weight of contamination would be lethal. In the particular experiment mentioned above, 89 mg of product killed a single mouse, 27 mg was not lethal and reduced liver catalase an average of 45% (3 mice), and 7 mg per mouse reduced liver catalase an average of 30% (3 mice). On the other hand, it is repeatedly noted in the toxohormone literature that acetone- or alcohol-precipitated tumor fractions (without manganese) are occasionally toxic. The toxicity we have noted may therefore have nothing to do with the manganese although the metal is suspect because of its known toxicity.

We hope to overcome this problem by the use of an improved method for the detection of traces of manganese.

2. We are not certain that this technique recovers all toxohormone. Again, it is repeatedly observed in the literature that toxohormone apparently occurs in more than one form. It has variously been stated to exist in free, protein-bound, polypeptide-bound, and nucleic acid-bound forms. It would seem unlikely that the manganese would precipitate all forms.

3. Preliminary evidence indicates that Mn will not precipitate toxohormone from the blood of tumor-bearing rats, whereas Kuzin et al.(4) state that they are able to prepare active fractions from such blood.

References

1. Greenstein, J. P. Biochemistry of Cancer. New York: Academic Press, 1954.

2. Feinstein, R. N., S. Berliner, and F. O. Green. Mechanism of inhibition of catalase by 3-amino-1,2,4-triazole. Arch. Biochem. Biophys. 76 32-44 (1958).

3. Nakahara, W., and F. Fukuoka. Toxohormone: A characteristic toxic substance produced by cancer tissue. Gann 40 45-69 (1949).

4. Kuzin, A. M., K. S. Sharukhova, and I. A. Chudinova. The effect of tumor extracts on the catalase and the coenzyme A of the liver of normal mice. Biokhimiya 20 126-8 (1955).

5. Kuzin, A. M., and I. A. Chudinova. The chemical nature of the toxin secreted by a malignant tumor. Doklady Akad. Nauk S.S.S.R. 107 289-290 (1956).

6. Ono, T., T. Sugimura, and M. Umeda. Preparation of potent concentrates of toxohormone free from nucleic acid. Gann 46 617-629 (1955).

7. Ono, T., M. Umeda, and T. Sugimura. Purification of toxohormone. Gann 48 91-100 (1957).

8. Greenfield, R. E., and A. Meister. The effect of injections of tumor fractions on liver catalase activity of mice. J. Natl. Cancer Inst. 11 997-1005 (1951).

PROGRESS REPORT: POTENTIATION OF TUMOR RADIOSENSITIVITY

Robert L. Straube and Harvey M. Patt

The theoretical basis for the suggestion[1,2] that high oxygen tension may be an important adjunct in radiotherapy of tumors stems from two conditions. First, an increase in oxygen tension above physiologic levels has a relatively small effect on the response of most normal tissues to irradiation. Second, many tumors are believed to be hypoxic relative to normal tissue. Thus it is inferred that there may be a differential gain in response in favor of a tumor when there is a general increase in oxygen tension at the time of irradiation. Although this sort of enhancement has been described in terms of a temporary regression for several tumors, its practical ramifications are not yet clear.

In an attempt to augment a differential gain in radiosensitivity, we are using various antimetabolites in conjunction with inhalation of oxygen at one atmosphere during local irradiation of tumors in vivo. Ten minutes before X-irradiation (1000 r) of solid Ehrlich ascites tumors 2-deoxy-d-glucose (0.5 ml 5% solution, intraperitoneally) was given with the anesthetized host (Nembutal) breathing air or oxygen at one atmosphere. The tumors, which had been implanted in the right thighs of CF No. 1 female mice at 7 or 8 weeks of age measured approximately 10 mm in each of two diameters at the time of irradiation. Inhalation of oxygen during irradiation increased initial tumor regression, in confirmation of the findings of others.[1-4] Although 2-deoxy-d-glucose appeared to enhance the oxygen response in preliminary experiments, this was not substantiated on further study. When the irradiation effect is evaluated in terms of the percentage of animals without palpable tumors at six weeks, there appears to be little difference between irradiation in air and irradiation in oxygen with or without 2-deoxy-d-glucose. Palpable tumors were found in approximately 30% of the survivors in all irradiated groups, in contrast to 70% in nonirradiated controls.

Irradiation under oxygen apparently increases the number of injured cells, which is manifest by early enhancement of tumor regression. This is presumed to be a consequence of the decrease in number of hypoxic tumor cells. On the other hand, irradiation under oxygen does not seem to modify the subsequent number of palpable tumors, a criterion which reflects the number of surviving cells rather than the number injured.

References

1. Churchill-Davison, I., C. Sanger and R. H. Thomlinson. Oxygenation in radiotherapy: Clinical applications. Brit. J. Radiol. 30 406-422 (1957).

2. Gray, L. H., A. D. Conger, M. Ebert, S. Hornsey and O. C. A. Scott. The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. Brit. J. Radiol. 30 638-648 (1953).

3. Hollcroft, J. W., E. Lorenz and M. Matthews. Factors modifying the effect of X-irradiation on regression of a transplanted lymphosarcoma. J. Natl. Cancer Inst. 12 751-762 (1952).

4. Hultborn, K. A., and A. Forssberg. Irradiation of skin tumors during pure oxygen inhalation. Acta Radiol. 42 475-484 (1954).

FRACTIONATION OF CHOLESTEROL ESTERS BY SILICIC ACID CHROMATOGRAPHY

Peter D. Klein and Erwin T. Janssen

A number of methods are available for analyzing the fatty acid composition of cholesterol esters, but all of these involve hydrolysis of the ester bond. In connection with our study of the metabolism of the individual cholesterol compounds, we have been interested in developing a method which would permit not only such analyses but also the separation of intact cholesterol esters.

A method of chromatography on silicic acid has been developed which permits the isolation of four types of esters: saturated, oleate, linoleate and arachidonate. While the saturated fatty acid esters are not individually resolved under the particular conditions employed, this fractionation provides the four main metabolic categories of interest to us. Recovery studies with model compounds show average recoveries of individual esters to be 91-100%, while total recoveries of mixtures averaged 94%. The standard error for an individual component is 16% of the value when the component is less than 10% of the total, and this error decreases to 3% when the component is 30% or more of the total

The method has been applied to the analysis of biological mixtures of plasma and liver esters of various species with widely differing compositions and has proved to compare favorably with other means of analysis involving hydrolysis.

HETEROGENEITY IN THE TURNOVER RATES OF RAT LIVER CHOLESTEROL ESTERS

Peter D. Klein and Rita A. Martin

Previously[1] we have reported a method for the fractionation of cholesterol esters into esters of saturated, oleic, linoleic and arachidonic acids. This technique has been used to study the rate of incorporation of newly synthesized cholesterol into its esterified forms.

When rats are injected with sodium acetate-1-C^{14} and sacrificed 30 min later, the specific activities of the four cholesterol ester fractions are different. At 4 hr after injection, the fractions are quite similar in specific activity. Statistical analysis of the results indicates a probability of less than .01 that the samples at 30 min are homogeneous, while after 4 hr this probability is greater than 0.2.

The data appear to substantiate a hypothesis that the rates at which cholesterol esters of rat liver are renewed are heterogeneous. The similarity of specific activities after 4 hr indicates that essentially equal proportions of each ester pool are renewed, and that the differences observed after 30 min are the reflections of individual rates of esterification.

These results, together with subsequent experiments, indicate that the esterification of cholesterol does not proceed via a single pathway for all esters, but that it is compartmentalized according to the fatty acid. The means by which such segregation occurs cannot be established as yet, but its existence may prove useful in explanations of the changes in serum cholesterol level when the composition of the dietary fat is altered.

We wish to thank Mr. Sylvanus Tyler for his invaluable aid in the statistical evaluation of the results.

Reference

1. Klein, P. D. and E. T. Janssen. Fractionation of cholesterol esters by silicic acid chromatography. This report, p.104

PROGRESS REPORT: THE BACTERIAL METABOLISM OF UNSATURATED FATTY ACIDS

II. The Utilization of Oleic-1-C^{14} Acid by *Lactobacillus arabinosus*

William M. O'Leary

The previous paper of this series[1] reported details of the bacterial utilization of *cis*-vaccenic (cis-11,12-octadecenoic) acid. It was shown that this compound, when supplied in lieu of biotin, is taken into the bacterial cells as such and is further converted to lactobacillic (*cis*-11,12-methylene-octadecanoic) acid.

This report will deal with the utilization of oleic acid by the same bacterial species. Oleic (*cis*-9,10-octadecenoic) acid differs structurally from the 11,12 configuration characteristic of the octadecenoic and cyclopropane nonadecanoic acids normally found in lactobacilli and other microorganisms.

Materials and Methods

Oleic-1-C^{14} acid was obtained from Tracerlab, Inc., Waltham, Mass. Since chromatographic analysis of a sample of this acid revealed no chemical or radiochemical impurities, it was used without further purification. For use in preparing culture media, sufficient highly purified unlabeled oleic acid was mixed with oleic-1-C^{14} acid to produce a specific activity of 50,150 cpm/μM.

The culture techniques and other experimental methods employed were those described in the previous report.[1]

Results

From a 12-liter culture of *L. arabinosus* grown on oleic acid in lieu of biotin, 5.8 g of dried bacterial cells were obtained from which 132.1 mg of fatty acids were extracted.

Microbiological assay of the medium after growth and removal of cells showed that of the 405 mg of oleic acid originally present, 317 mg remained, indicating that 88 mg or 0.312 mM had been utilized during growth of the bacteria.

Results of the analysis of fatty acids extracted from the bacteria appear in Table 22. The combined total cellular content of octadecenoic and cyclopropane nonadecanoic acids was 0.272 mM, or approximately

TABLE 22

Analysis in duplicate of the fatty acids of L. arabinosus grown on oleic-1-C^{14} acid (50,150 cpm/μM)*

Acid	Percentage of total cellular fatty acids		Specific activity, cpm/μM		Total content, mM	
Octadecenoic	26.6	27.1	42,630	42,400	0.124	0.128
Capric	1.5	2.1	**			
Lauric	2.8	2.3	**			
Myristic	2.9	3.0	**			
Palmitic	32.2	33.1	990	1,010		
Cyclopropane nonadecanoic	33.2	32.1	41,820	41,570	0.148	0.143
Totals	99.2	99.7			0.272	0.271

*0.312 mM removed from medium during growth.

**Not significantly above background.

87% of the amount of oleic acid apparently removed from the medium during growth. Measurements of the specific activities of the fatty acid fractions and of the nonsaponifiable material showed that radioactivity was largely limited to the octadecenoic and nonadecanoic acids with little or no activity in other lipid fractions. The specific activities of these two acids were similar and were approximately 85% of that of the oleic acid in the medium.

It was desired to obtain at least some indication of the positional isomers present in the fatty acids of bacteria grown on oleic (cis-9,10-octadecenoic) acid. Microbiological assay and melting-point determinations of the cyclopropane nonadecanoic acid present in the bacteria gave data similar to those reported for lactobacillic (cis-11,12-methylene-octadecanoic) acid[2,3] and were not in agreement with those reported for the 9,10 isomer of the same compound.[4] Since the unsaturated fatty acids had to be converted to their biologically inactive dihydroxy derivatives in order to be chromatographically separable, they could not be identified by microbiological methods. The usual separation and analytical data for the isomers and their derivatives are too similar to be informative, and not enough material was available for degradation studies. However, it was possible to perform mixed melting point determinations with these dihydroxy acids using various authentic samples. These data appear in Table 23. These preliminary studies indicate that in cells of L. arabinosus grown on oleic acid, the 9,10 isomer, the octadecenoic and cyclopropane nonadecanoic acids present are of the 11,12 configuration.

TABLE 23

Mixed Melting Point Determinations

	Compound	Melting range, °C
I	11,12-Dihydroxy-octadecanoic acid (prepared from cis-vaccenic acid)	92.7 - 93.8
II	9,10-Dihydroxy-octadecanoic acid (prepared from oleic acid)	92.0 - 93.4
III	Dihydroxy-octadecanoic acid from cells grown on cis-vaccenic acid	93.0 - 94.0
IV	Dihydroxy-octadecanoic acid from cells grown on oleic acid	92.0 - 93.9
	I + III, 1:1	92.0 - 93.6
	I + IV, 1:1	91.6 - 93.4
	II + III, 1:1	80.0 - 84.2
	II + IV, 1:1	81.7 - 86.0
	III + IV, 1:1	91.7 - 94.0

Discussion

In L. arabinosus cells grown on complete synthetic medium containing biotin, the sole octadecenoic acid is cis-vaccenic acid, and the sole cyclopropane nonadecanoic acid is lactobacillic acid, both 11,12 isomers.[5] When biotin is replaced in a medium by cis-vaccenic acid, the acid is incorporated into the bacterial cell where it constitutes 95% of the cellular content of this compound, and where it further serves as the precursor of lactobacillic acid.[1] These observations were in agreement with previously postulated pathways of unsaturated fatty acid biosynthesis in bacteria.[6,7] However, the metabolic functions of other biotin-replacing positional isomers of octadecenoic acid such as oleic acid were still obscure.

The experiments reported here show that octadecenoic acid supplied in the medium in the form of oleic acid, the 9,10 isomer, is also incorporated into bacterial cells, and in turn serves as the precursor for a cyclopropane nonadecanoic acid. Of major interest are the results of determinations of microbiological activity and mixed melting points which indicate that in cells grown on this 9,10 isomer, the octadecenoic and cyclopropane nonadecanoic acids present are themselves 11,12 isomers. More detailed analyses requiring larger amounts of cellular acids are necessary to

establish unequivocally the exact location of the double bond and the cyclopropane ring in these compounds. These preliminary observations do strongly suggest that the 9,10 isomer supplied in lieu of biotin is isomerized to the 11,12 forms characteristic of fatty acids found in so-called normal lactobacilli lipids, i.e., lipids from lactobacilli grown on biotin rather than biotin substitutes.

The further function of lactobacillic acid in bacterial physiology, which may provide a clue to the reason for this marked preference for 11,12 isomers, is now being investigated.

References

1. O'Leary, W. M. Progress report: The bacterial metabolism of unsaturated fatty acids. I. The utilization of cis-vaccenic acid by Lactobacillus arabinosus. Biological and Medical Research Division Semiannual Report. Argonne National Laboratory, ANL-5916 (1958). pp. 145-147.
2. Hofmann, K. and C. Panos. The biotin-like activity of lactobacillic acid and related compounds. J. Biol. Chem. 210 687-693 (1954).
3. Hofmann, K. and R. A. Lucas. The chemical nature of a unique fatty acid. J. Am. Chem. Soc. 72 4328 (1950).
4. Yoho, C. W. The synthesis of long-chain fatty acids containing a cis cyclopropane ring. Dissertation, University of Pittsburgh (1957).
5. Hofmann, K., R. A. Lucas, and S. M. Sax. The chemical nature of the fatty acids of Lactobacillus arabinosus. J. Biol. Chem. 195 473-485 (1952).
6. Potter, R. L., and C. A. Elvehjem. Biotin and the metabolism of Lactobacillus arabinosus. J. Biol. Chem. 172 531-537 (1948).
7. O'Leary, W. M., and K. Hofmann. Short-chain monoethenoid fatty acids as possible precursors in bacterial biosynthesis of cis-vaccenic acid. Fed. Proc. 16 228 (1957).