ANL-6368
Biology and Medicine
(TID-4500, 16th Ed., Amended)
AEC Research and Development Report

ARGONNE NATIONAL LABORATORY
9700 South Cass Avenue
Argonne, Illinois

BIOLOGICAL AND MEDICAL RESEARCH DIVISION SUMMARY REPORT

January through December, 1960

May 1961

Preceding Report:
ANL-6264 July through December, 1959

Operated by The University of Chicago
under
Contract W-31-109-eng-38
with the
United States Atomic Energy Commission

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TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

ACUTE RADIATION MORTALITY IN THE PARAKEET

S. P. Stearner, S. A. Tyler, M. H. Sanderson and E. J. Christian

Introduction

Our studies of comparative radiobiology in the bird have confirmed the existence of dose-reductive processes that influence the acute mortality and pathological responses following an exposure to ionizing radiation. In addition, statistical appraisals of the interdependence of the observed mortality and the amount and duration of the radiation revealed quantitative information of importance in determining the nature and kinetics of the injury processes underlying the acute syndrome. In some respects, different avian species exhibit distinctly contrasting responses to an acutely lethal insult. One group that includes the chick and the duck expresses an injury as mortality within 48 hours after exposure.[1,2] A second group that includes the pigeon, the parakeet, and the canary does not show this initial response.[3,4] Much information is available to suggest that injury processes that terminate as 0 to 2-day mortality in one group are also active in the second group, but the injury accumulated does not result in lethality under comparable exposure conditions. For each species, however, varying the exposure time from a few minutes to 24 hr produces marked changes in the probability and/or the distribution of mortality within the 3- to 30-day postirradiation period.

This report will summarize the relations between dose and exposure time in the parakeet following Co^{60} γ-irradiation. Mortality within 30 days is used as the measure of sustained insult, and the similarities between empirical descriptions of injury accumulation processes for the parakeet and chick are pointed out.

Method

Male and female parakeets (Melopsittacus undulatus) 6 to 8 weeks old were obtained from Steves Aviary, San Francisco, California. These birds, raised under the supervision of Karl F. Meyers, University of California, had received aureomycin-medicated feed for 2 weeks prior to shipment as preventive treatment against possible latent psittacosis infection. In our animal quarters, the birds were housed in flight cages, 15 per cage, and fed a millet-canary seed mixture supplemented with cod-liver oil. The birds were kept on the animal farm for two weeks before irradiation. Conditions of exposure to Co^{60} γ-rays were the same as have been described for the chick.[2] Gamma-ray doses ranged from 1400 to 3400 r, delivered over periods varying from 4 min to 24 hr. In most instances there were 30 to 36 parakeets in each dose/exposure-time group, and a total of 2413 birds were irradiated.

Results

Radiation deaths began to appear about 4 days after exposures and maximum frequency occurred between days 10 and 11. The frequency distribution of daily mortality remained unchanged as exposure time was varied. This may be seen by comparing daily mortalities for exposures of 15 min and 24 hr as given in Table 1. The dose-mortality relations for exposure periods of 4 min to 24 hr were determined. Mortality in groups exposed for 4-, 15-, and 30-min periods was not distinguishably different, and the average values for these groups were used to represent the 15-min exposure group. Protraction of the exposure over periods longer than 8 hr produced little change in dose effectiveness; therefore, 8-, 16-, and 24-hr exposures were also combined.

TABLE 1

Daily mortality in the parakeet (both sexes) for exposures of 15 min and 24 hr

Exposure time, min	Dose, r	N	Days after irradiation																												Survivors at 30 days
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	
15	1400	30								2		1												1		1							25
	1500	30				1			1	1			3			1		1		1	2	1											18
	1600	29						3	4		3	2	1	1	2	2		1															10
	1700	30			1			1	1		2	3	2	2	3	1		1	2	1		1											9
	1800	30				1			1	4	3	2	2	2	1	2		1	2		1			1						1	1		5
	1900	30						2	1	3	1	3	1	2	3	4	2				2		1		1								4
	2000	30				1		3	3	3		1	3	3	2	1	1	1					1	1				1					5
	2100	30					1	2	2	3	2	5	3	2	1	2	2			1	2	1											1
	2200	36					1	2	3	6	1	5	3	5	4	1	1				1						2						1
1440	1800	30	1						1	1		1			3																	23
	2000	29								2						1		1	1	1													23
	2200	29					1	1		3	2	2	1		3		1	1					2										12
	2400	30					1			1		4	5	1			1	2	1	1		1	1										11
	2600	30					1		2	2	5	1	1	1	2	3	2																10
	2800	30		1			1	2	3	1	3	3	3	2		1	1	1	1														7
	3000	29		1			1		2		2	3	4		2	1	2	1															10
	3200	30						3	1	1	5		2	5	2	2		1	2		2												4
	3400	30				1	1	2	2	3	2	2	7	3	4	1		1		1													0

The 30-day mortality data and the dose-mortality curves for different times of exposure are presented in Figure 1. With increase in exposure time through 8 hr, the probit curves show an increase in LD_{50} and a decrease in slope. For exposures of 8 hr or longer, the dose-mortality pattern is assumed to have reached a stable and unvarying configuration that represents uniquely the lethal results of a time-independent (irreversible) effect. The LD_{50} for the irreversible component is 2310 r ($\hat{\sigma}$ = 632 r). If the action of this effect is independent of time-dependent (reversible) processes, its contribution can be subtracted from the total mortality. The resultant mortality in groups exposed for shorter periods then represents the time-dependent component. Such a partition has been achieved by applying the following formula:

$$Q = q + (1 - q)p \quad ; \quad q = \frac{Q - p}{1 - p}$$

where Q is the total proportion dying, q, is the proportion dying of the reversible effect and p is the proportion that succumb to the irreversible effect. The resultant mortality, q represents the reversible effect(s) expressed within the 30-day postirradiation period (Figure 2). Dose-exposure time relations for the 20%, 50% and 80% mortality levels (Figure 3) are approximately linear with respect to exposure time, and each equimortality category yields approximately equal ratios of intercept to slope. This ratio, with dimension of time and average value of 287 min, characterizes the action of reversible processes in the parakeet.

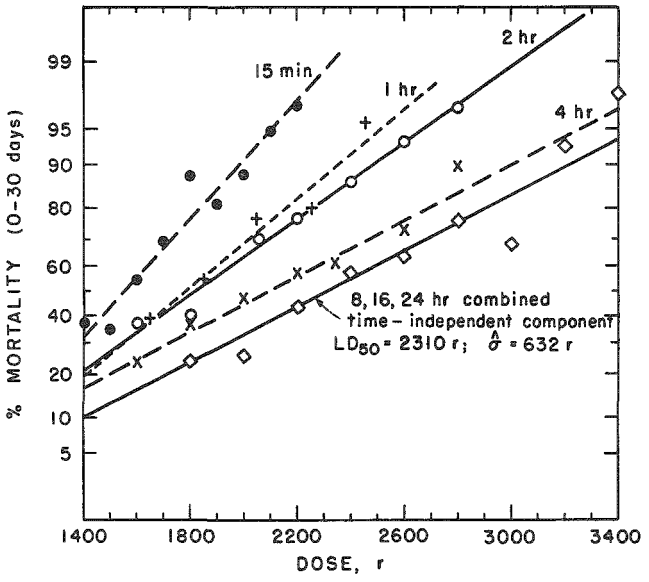

Figure 1

Effect of exposure time on dose-mortality relations

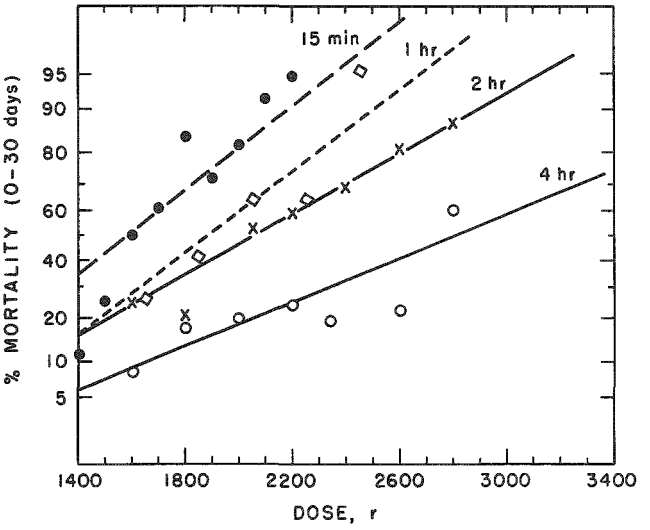

Figure 2

Mortality within 30 days postirradiation resulting from time-independent processes only (reversible effect)

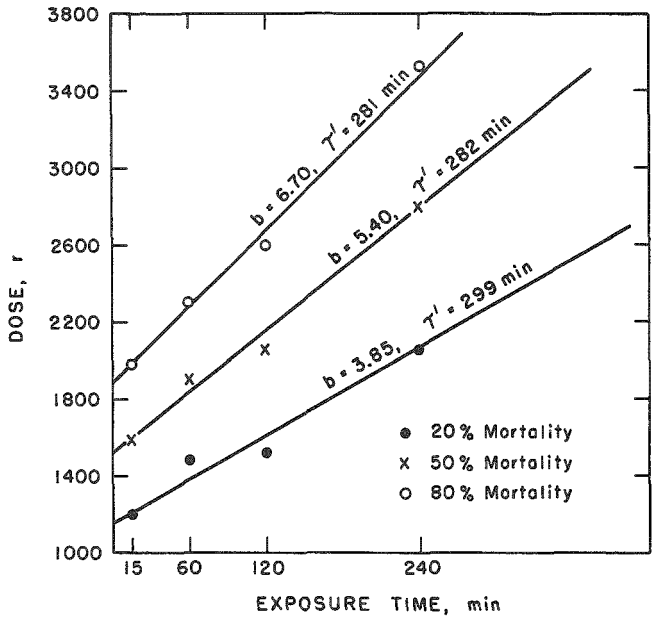

Figure 3

Dose-exposure time relation for rate dependent effect at 20, 50, and 80% mortality levels, as interpolated from mortality curves in Figure 2

The presence of a single mortality peak during the 30-day post-irradiation period precludes separation of distinct lethal mechanisms on the basis of time of death in a manner similar to that employed for the chick and chick embryo.[2,5,6] Although the influence of injury processes with different kinetics must be assumed, a distinguishing pathology associated with distinct injury processes is absent in the parakeet. Thus, in order to investigate the kinetics of individual lethal processes that contribute to the total mortality pattern, some rule descriptive of the combined action is essential. Here, we have assumed that the two major classes of injury mechanisms (reversible and irreversible processes) are independent in action. One then might ask if there is some justification for this choice of model. In studies of dose/exposure-time relations for acute radiation mortality in the chick embryo[5,6] and the chick,[7] experimental results supporting the validity of this model are:

1. In the chick embryo, mortality after the initial period is shown to include a reversible component that is separable on the basis of time and dose-range of expression. Because the dose ranges for reversible and irreversible processes operating in this period are almost completely distinct, it is not necessary to correct the reversible effect for contribution of irreversible processes. The dose/exposure-time relations for this effect indicate the presence of a linear reversal mechanism, the characterizing constant, τ, of which is equal to 265 min.

2. In the chick, similar analysis of the data also reveals a reversible effect expressed over the same time period, but in this case the dose range of irreversible processes is not entirely distinct. Separation of reversible and irreversible components by the method used for the parakeet yields a reversible component with characterizing ratio, τ, equal to 254 min.

The reversible lethal process expressed within the first 1 or 2 days after irradiation in the chick embryo and the chick is not present in the parakeet. For radiation lethality expressed after the initial period, however, the characterizing constants of the reversible processes in these subjects are statistically equivalent, i.e. τ = 265 min, 254 min and 287 min for the chick embryo, chick and parakeet respectively. The standard error associated with these averages is approximately 40 min.

Summary

Dose-mortality curves for the parakeet exposed to Co^{60} γ-radiation indicate that a linear reversal mechanism with a time constant of 287 min and an irreversible component with LD_{50} equal to 2310 r are the predominant injury processes operating within 30 days after irradiation. Radiation deaths rarely occur before the fourth post-irradiation day. These findings together with the similarity between time constants found for parakeet, chick and chick embryo, suggest that the reversible process in the parakeet may be the same as corresponding processes expressed in the chick and chick embryo. Independence of injury mechanisms was assumed before a separation of effects could be accomplished in the parakeet.

References

1. Stearner, S. P. The effect of variation in dosage rate of roentgen rays on survival in young birds. Am. J. Roentgenol. Radium Therapy 65 265-71 (1951).

2. Stearner, S. P., and S. A. Tyler. An analysis of the role of dose and dosage rate in the early radiation mortality of the chick. Radiation Research 7 253-66 (1957).

3. Stearner, S. P., M. H. Sanderson and E. J. Christian. The initial post-irradiation period in the pigeon. Biological and Medical Research Division Semiannual Report, Argonne National Laboratory ANL-5732 (1957), pp. 65-67.

4. Stearner, S. P. Unpublished results.

5. Stearner, S. P., M. H. Sanderson, E. J. Christian and S. A. Tyler. Modes of radiation death in the chick embryo. I. Acute syndromes. Radiation Research 12 286-300 (1960).

6. Tyler, S. A., and S. P. Stearner. Modes of radiation death in the chick embryo. II. A model of lethal mechanisms. Radiation Research 12 301-316 (1960).

7. Tyler, S. A., and S. P. Stearner. Unpublished results.

PROGRESS REPORT: FERTILITY OF CF NO. 1 FEMALE MICE IRRADIATED WITH FISSION NEUTRONS

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

In these experiments, CF No. 1 female mice were exposed to fission neutrons at the CP-5 reactor and are now being tested for fertility at intervals of about 2 months. The results of the first 3 tests were reported previously.(1)

Table 2 gives the results from all tests to date for mice exposed to single, whole-body doses of 20, 50, 75, 100, and 150 rads of fission neutrons at approximately 5 rads/min.

It is now clear that the lowest dose of 20 rads produces a significant decrease both in the number of pregnancies and in litter size. Injury is not immediately apparent, but the fourth through the sixth tests indicate that it progresses with time. Complete sterility is produced at all other doses.

TABLE 2

Fertility in CF No. 1 female mice after exposure to fission neutrons

Dose, rads	Test No.	No. mice	Pregnancies		Avg. litter size	Group mean progeny	
			No.	%		Total	Per mouse
Control	1	20	19	95	10.5	1113	55.6
	2	20	20	100	12.2		
	3	20	20	100	10.2		
	4	19	19	100	10.3		
	5	17	15	88.2	9.1		
	6	15	13	86.6	8.1		
20	1	15	15	100	10.4	639	42.6
	2	15	15	100	10.6		
	3	14	13	92.8	10.9		
	4	14	13	92.8	8.8		
	5	14	7	50.0	7.4		
	6	12	3	25.0	5.3		
50	1	16	14	87.5	7.6	159	9.9
	2	16	12	75.0	3.7		
	3	15	3	20.0	2.7		
	4	15	0	0	0		
	5	15	0	0	0		
	6	15	0	0	0		
75	1	16	14	87.5	7.6	116	7.2
	2	16	3	19.0	3.3		
	3	16	0	0	0		
	4	16	0	0	0		
	5	15	0	0	0		
	6	15	0	0	0		
100	1	11	11	100	6.4	70	6.4
	2	11	0	0	0		
	3	11	0	0	0		
	4	10	0	0	0		
	5	9	0	0	0		
	6	8	0	0	0		
150	1	16	7	44.0	3.6	25	1.5
	2	16	0	0	0		
	3	16	0	0	0		
	4	14	0	0	0		
	5	9	0	0	0		
	6	6	0	0	0		

Although the reproductive system is damaged to some extent immediately following the smaller neutron doses, as seen by the reduced litter size, complete sterility does not occur at once. The first mating test was successful even at the highest dose (150 rad). Complete sterility occurs progressively earlier with increase in total dose.

Reference

1. Vogel, H. H. and D. L. Jordan. Fertility of CF No. 1 female mice irradiated with fission neutrons. Biological and Medical Research Division Semiannual Report, Argonne National Laboratory. ANL-6264 (1959), pp. 31-32.

THE COMPARATIVE EFFECTIVENESS OF AET AS A PROTECTIVE AGENT AGAINST MORTALITY IN NEUTRON- AND γ-IRRADIATED MICE

D. L. Jordan, H. H. Vogel, Jr., N. A. Frigerio,
N. Bink, and R. Barhorst

A previous report[1] presented data on pretreatment of CF No. 1 female mice with AET (S, 2-aminoethylisothiuronium bromide hydrobromide) administered orally. The present report summarizes 9 neutron and 8 γ-ray experiments in which AET was tested for chemical protection against acute mortality after fission neutrons and Co^{60} γ-rays. AET-treated mice received 0.34-0.5 cc (approximately 600 mg/kg of body weight) in acetate buffer at pH 7.4.

Nine groups, a total of 95 control mice and 191* AET-treated mice, were exposed to 350 rads of fission neutrons, which at the CP-5 reactor facility is approximately the $LD_{90/30}$ level. This irradiation took 60 to 70 minutes. The results are given in Figure 4. AET-pretreatment resulted in 39% mortality. This corresponds to the mortality for a neutron exposure of approximately 315 rads for untreated mice[2] and thus represents a dose reduction of 35 rads or 10%. Eight groups, a total of 71 control mice and 111 AET-treated mice, were exposed to Co^{60} γ-rays at a similar dose level (1100 rads = $LD_{90/30}$). Here the mortality of the AET-treated mice was 22%. This was considered effective protection since the exposure time was in excess of 2 hr. Mortality corresponds to that for a single dose (Co^{60} γ) of 813 rads, a dose reduction of 287 rads or 26%. It is thus apparent that AET, like cysteine[3] and serotonin,[4] is more effective as a protective agent against γ- and X-rays than against fission neutrons.

The mean survival time of the 30-day decedents is tabulated below; the numbers in parentheses represent the number of mice that died in 30 days per number irradiated.

	Mean survival time, days
γ-irradiated controls (63/71)	13.0
AET-treated, γ-irradiated (25/111)	13.1
Neutron-irradiated controls (85/95)	8.2
AET-treated, neutron-irradiated (67/170)	8.6

*Twenty-one mice pretreated with AET and irradiated with neutrons were eliminated from the data presented in Figure 4 because they died of chemical toxicity within 48 hr of irradiation. If these animals were counted, the 30-day mortality would be increased from 39.4 to 46.0%. Four mice that died from AET toxicity in the γ-ray experiments were similarly omitted from the data of Figure 4.

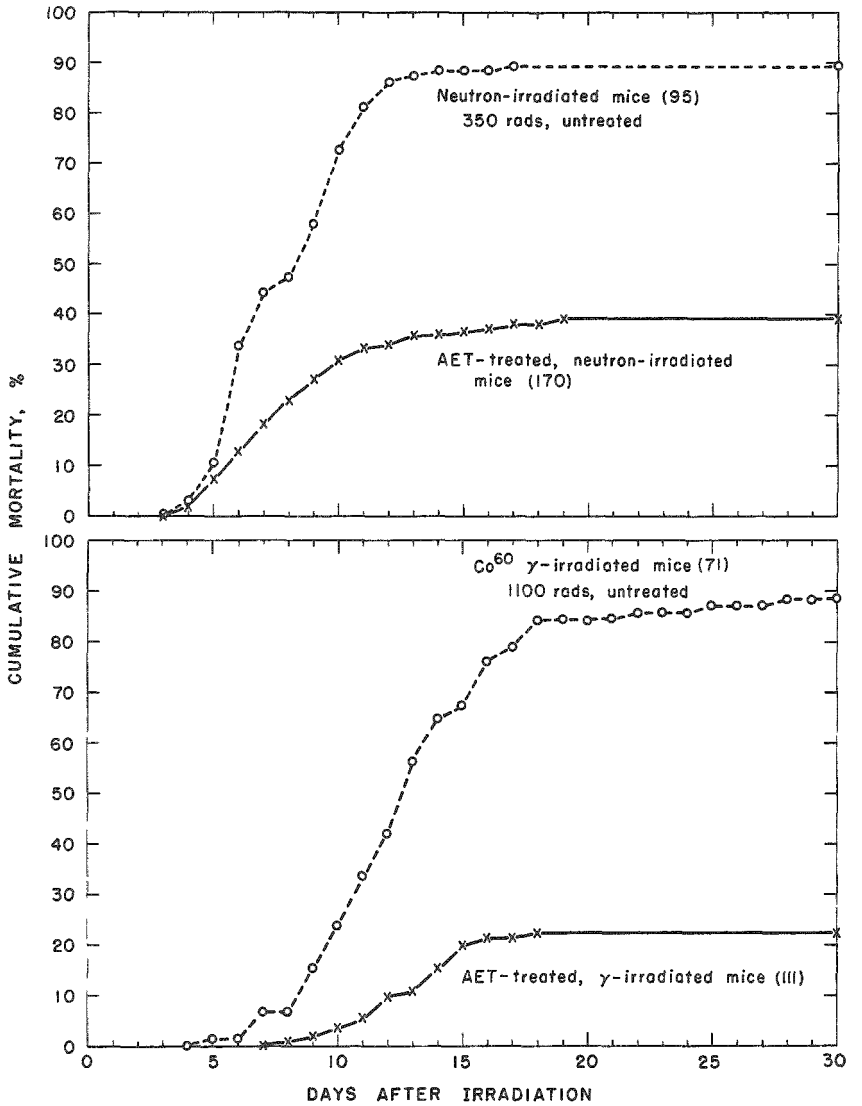

Figure 4. Comparative protective action of AET in mice irradiated with fission neutrons or Co^{60} γ-rays

Thus, it appears that the pretreatment with AET does not significantly change the mean survival time of those mice dying in the acute 30-day period after either γ- or neutron irradiation.

It had been assumed that oral administration of AET would be more effective if pretreatment was initiated in the absence of food in the stomach[5] to promote more rapid absorption. This assumption was tested by withholding food for 4 hr prior to AET administration and subsequent irradiation. When food was withheld, the mortality of the AET-treated, starved animals was 54%; however, in the AET-treated animals allowed food it was only 17% (Table 3). Fission neutron exposure was, as before, 350 rads ($LD_{90/30}$). Of special interest is the pattern of daily death. Deaths occurring on the first and second day are usually a result of chemical toxicity of the AET. The data indicate that the presence

of food may act to reduce absorption rate by the gut and thus render the animal less sensitive to the toxic action of the AET. Fourteen of these toxic deaths occurred in the starved group in contrast to three in the group of animals with food present in the stomach.

TABLE 3

Effect on daily deaths of withholding food for 4 hr before pretreatment with AET. All mice (CF No. 1 female) were given 350 rads ($LD_{90/30}$) of fission neutrons.

Day*	Food allowed		Food withheld	
	AET-treated (48)**	Irradiated controls (16)	AET-treated (48)	Irradiated controls (16)
1	3		8	
2			6	
3				
4				
5			1	1
6			3	1
7	1	2	2	
8		1	3	1
9	1	2		2
10		3	1	5
11		4		2
12		1		4
13	1	1	1	
14				
15	1			
16				
17		1	1	
18				
19	1			
	8/48	15/16	26/48	16/16
Mortality	16.6%	93.8%	54.2%	100%

*No deaths were observed from day 20 through day 30.

**Number of animals.

When deaths due to chemical toxicity are eliminated from the mortality figures, the remaining deaths can be assumed to be due to irradiation effect alone. Mortality is then reduced to 35% (12/34) for the unfed animals and is increased only slightly to 18% (8/45) for the fed group.

It would seem from these figures that the withholding of food before oral administration of AET does not increase the effectiveness of the radioprotective action of this agent, but may increase its toxicity.

References

1. Vogel, H. H., Jr., N. A. Frigerio, and D. L. Jordan. Chemical protection: The comparative effectiveness against fission neutrons and Co^{60} gamma rays of AET administered orally to mice before irradiation. Biological and Medical Research Division Semiannual Report, Argonne National Laboratory. ANL-6200 (1959), pp. 47-48.

2. Jordan, D. L., and H. H. Vogel, Jr. Thirty-day survival of female mice and rats given single whole-body exposures to fission neutrons. Biological and Medical Research Division Semiannual Report, Argonne National Laboratory. ANL-6093 (1958), pp. 63-64.

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

4. Vogel, H. H., Jr., N. A. Frigerio and D. L. Jordan. Prophylactic and therapeutic treatment of neutron-irradiated mice. Radiation Research 12 483 (1960).

5. Maisin, J. Personal communication (1959).

LIFE SHORTENING IN MICE IRRADIATED WITH EITHER FISSION NEUTRONS OR Co^{60} γ-RAYS AT LOW DOSE RATES

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

In previous work, life shortening was compared in mice given 13 daily exposures to fission neutrons or Co^{60} γ-rays at relatively high dose-rates.(1) The present experiment was carried out to test the effects of decreased dose rate of 13 daily exposures to two radiations. Female CF No. 1 mice were exposed to daily doses of either fission neutrons or Co^{60} γ-rays at dose rates of approximately 1 rad/min. This low intensity was achieved by increasing the distance between the 18 Co^{60} sources and the animal position in the gamma-neutron radiation chamber,(2) or by closing completely a boral filter plate located between the CP-5 reactor and the uranium converter plate.(3) Five groups of mice were given 13 brief daily exposures (3.75, 7.5, 15, 30, and 60 min) to each radiation, and the mean survival time after radiation was compared with that for unirradiated control mice.

The experimental plan, radiation data, mean survival time after first exposure of the various groups, and reduction of life span are given in Table 4.

TABLE 4

Longevity data for 13 daily exposures to fission neutrons or Co^{60} γ-rays at low dose-rates

No. of animals	Daily dose, rads	Total dose, rads	Mean survival time, days, ± S.E.	Longevity, % of control	Reduction in life span, %
Fission neutrons, 1 rad/min					
51	-	-	486.3 ± 22.9	100	
31	3.3	44	445.7 ± 24.6	92	8
31	7	87	457.7 ± 26.6	94	6.0
30	13	174	375.9 ± 23.7	78 6	21.4
32	27	349	240.8 ± 16.1	50.4	49.6
30	54	698	23.2 ± 3.9	4.8	95.2
Co^{60} γ-rays, 1 rad/min					
48	-	-	493.1 ± 23.8	100	
40	4.3	56	473.6 ± 17.0	96	4.0
40	8.6	111	485.8 ± 20.4	98.3	1.7
40	17	222	445.4 ± 22.5	90.3	9.7
39	34	444	331.7 ± 24.4	67.3	32.7
40	68	888	268.3 ± 28.4	54.4	45.6

A comparison of the effects of the 13 daily doses on life span of the irradiated mice is plotted in Figure 5. Fission neutrons were 2 or 3 times as effective as Co^{60} γ-rays in reducing the life span by 50% (950 rads, γ; 350 rads, neutrons).

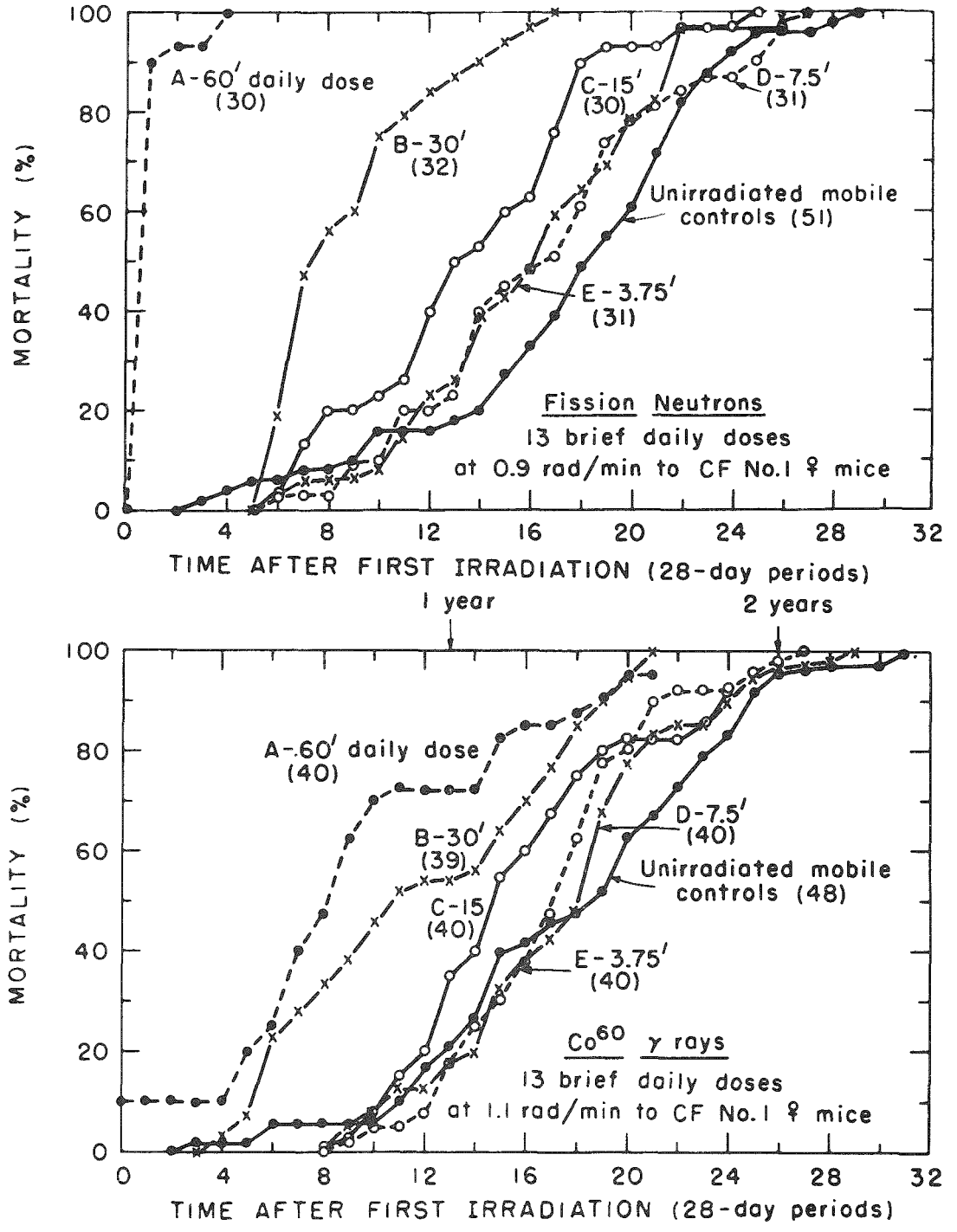

Figure 5. Cumulative mortality in CF No. 1 female mice after 13 daily exposures to small doses of fission neutrons or Co^{60} γ-rays. The mean survival time is given in Table 4 for each group.

This RBE figure (γ/n) does not differ significantly from that obtained by comparing the acute $LD_{50/30}$ doses after single exposures to the two radiations.[4]

If one compares the results of this experiment, carried out at the low intensity of 1 rad/min, with a previous experiment at higher dose rates (Table 5), it becomes clear that the dose rate is much more important for γ-radiation than for neutrons. This earlier work[1] indicated that a dose of 300 to 350 rads of fission neutrons (at 4 to 5 rads/min) was required to cut the mean survival time in half. When the neutron dose rate is decreased to only 0.9 rad/min (Figure 5), approximately 350 rads are still needed to reduce longevity by 50%. It is clear, therefore, that the effect on life span of fission neutron irradiation in the range of intensities studied is relatively independent of the neutron dose rate. These data appear to confirm earlier work in which we reported that the acute $LD_{50/30}$ after single irradiations of mice with fission neutrons did not change significantly, whether the exposure was carried out in 1.5 or 24 hours.[5]

TABLE 5

Longevity data for 13 daily exposures to fission neutrons or Co^{60} γ-rays at relatively high dose rates

No. of animals	Daily dose, rads	Total dose, rads	Mean survival time, days, ± S.E.	Longevity, % of control	Reduction in life span, %
Fission neutrons, 4-5 rads/min					
36	0	0	601.4 ± 24.7	100	
36	2.2	29	562.6 ± 21.1	99.1	0.9
18	4.4	57	459.3 ± 31.1	80.8	19.2
36	9.0	117	381.9 ± 23.8	67.3	32.7
18	17.5	227	377.1 ± 40.2	66.4	33.6
36	36	468	188.9 ± 17.9	33.3	66.7
18	70	908	16.0 ± 0.3	2.8	97.2
Co^{60} γ-rays, 13 rads/min					
32	0	0	594.5 ± 26.2	100	
24	6.3	81	516.5 ± 30.8	86.7	13.3
18	13	171	416.6 ± 33.3	70.1	29.9
24	25	351	406.3 ± 34.8	68.2	31.8
18	52.5	682	222.6 ± 31.7	37.5	62.5
24	108	1406	49.2 ± 12.2	8.4	91.6
18	210	2727	14.3 ± 0.23	2.4	97.6

On the other hand, if one compares the results of shortening of the mean life span following γ-radiation at the two intensities used - 13 rads/min[1] and 1.1 rad/min (Figure 5), it is evident that dose rate of γ-rays has a marked effect: At the higher dose rate, a total exposure of only 400 to 500 rads is necessary to shorten the mean life span by 50%. When the rate was decreased to only 1 rad/min this 50% level was not reached even at the highest total dose used (888 rads).

If we compare the data on reduction of life span after γ-radiation at the low dose rate (last column, Table 4) with similar figures for the high dose rate, (Table 5), it is apparent that the low intensity is much less effective in shortening the life span in these mice. It is clear from Figure 6 that total γ-ray doses up to 222 rads (at 1 rad/min) do not significantly reduce life span (at least by 10%), whereas a total dose of 171 rads (at 13 rads/min) reduced the life span by almost a third.[1]

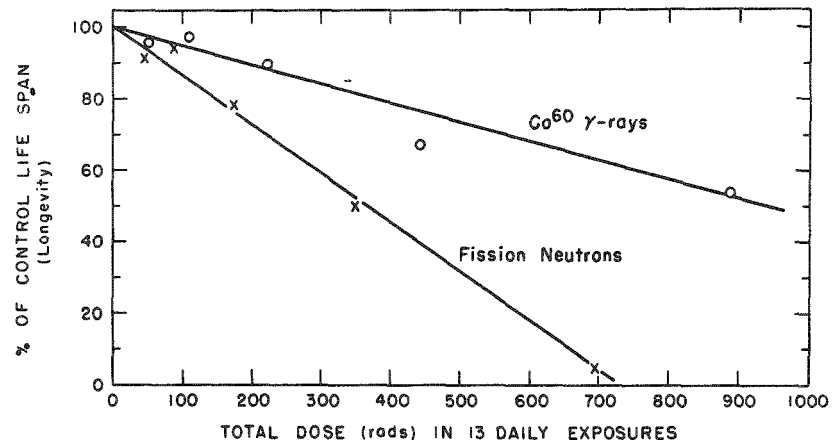

Figure 6. Influence of total dose on longevity. Longevity is plotted against total dose (rads) for 13 daily exposures of fission neutrons or Co^{60} γ-rays, delivered at 1 rad/min.

If one plots the total dose of neutrons delivered at either dose rate against reduction in life span, a single curve fits the points from both neutron experiments equally well (data in Tables 4 and 5). However, a similar comparison between the two γ-ray experiments (13 rads/min vs 1 rad/min) indicates two completely different curves and illustrates again the dose-rate dependence of γ-radiation.

References

1. Vogel, H. H., Jr. and D. L. Jordan. Pilot study of subacute irradiation for future fast neutron and gamma-ray exposures. II. Longevity after 13 daily exposures. Semiannual Report of Biological and Medical Research Division, Argonne National Laboratory. ANL-6200 (1959), pp. 51-55.

2. Vogel, H. H., Jr., R. A. Blomgren, and N. J. G. Bohlin. Gamma-neutron radiation chamber for radiobiological studies. Nucleonics 11 (3) 28-31 (1953).

3. Vogel, H. H., Jr., J. W. Clark, and D. L. Jordan. Modification of the gamma-neutron radiation chamber for use at the Argonne Research Reactor. Report on Biological, Medical, and Biophysics Programs, Argonne National Laboratory. ANL-5288 (1954), pp. 35-36.

4. Jordan, D. L. and H. H. Vogel, Jr. Thirty-day survival of female mice and rats given single whole-body exposures to fission neutrons. Biological and Medical Research Division Semiannual Report, Argonne National Laboratory. ANL-6093 (1958), pp. 63-64.

5. Vogel, H. H., Jr., J. W. Clark, and D. L. Jordan. Comparative mortality after 24-hour, whole-body, exposures of mice to fission neutrons and cobalt-60 gamma rays. Radiation Research 6 460-468 (1957).

LACK OF ACQUIRED RADIORESISTANCE AFTER SINGLE DOSES (50 RADS) OF FISSION NEUTRONS OR Co^{60} γ-RAYS

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

A number of experiments have indicated that mice appear to develop significant radioresistance following an initial exposure to small doses of X-radiation. The subject has recently been reviewed by Dacquisto.(1) It seemed of interest to test this hypothesis when the first small dose was either fission neutrons or Co^{60} γ-rays.

The plan of the experiment is illustrated in Table 6. Sixteen groups each consisting of 24 to 32 young adult female CF No. 1 mice (406 total) were given 50 rads of either γ-rays or fission neutrons (single brief whole-body exposures) followed at four different intervals (1, 2, 3, and 10 weeks) by a single midlethal dose of one of the two radiations. Each of the 16 experimental groups had its own age-control group (406 total), which received no preliminary exposure, but was given the second midlethal exposure together with its experimental group.

TABLE 6

Plan of experiment

Group	Exposure No. 1 (50 rads)	Interval, weeks	Exposure No. 2 (320 rads neutrons or 900 rads γ-rays)
1	neutrons	1	neutrons
2		2	
3		3	
4		10	
5	neutrons	1	γ-rays
6		2	
7		3	
8		10	
9	γ-rays	1	neutrons
10		2	
11		3	
12		10	
13	γ-rays	1	γ-rays
14		2	
15		3	
16		10	

If significant radioresistance were actually acquired by a small preliminary exposure, then there should be decreased mortality among the "experimental" mice exposed to the second, larger dose of radiation when compared with the acute mortality of their age controls, which received the second dose only.

The results of the experiment are summarized in the bar graphs of Figure 7. Of the 16 groups tested, 12 experimental groups exhibited 30-day mortality as high as or higher than that of their respective age controls. In no case was there a significantly lower mortality among the experimental animals compared to their own controls. In 4 of the 16 groups the mice that received 50 rads prior to the second lethal exposure showed a significantly higher 30-day mortality than their controls. Fisher's exact test was used for the difference between experimental and control proportions dead.

The data from this experiment give no clear-cut evidence for acquired radioresistance (as measured by acute, 30-day mortality) in the mice irradiated with 50 rads of either fission neutrons or Co^{60} γ-rays.

In Figure 8 are illustrated the 30-day mortality data of the four experimental groups that showed significantly higher mortality than their respective age controls. It is of interest that in three of these four cases, the preliminary dose was 50 rads of fission neutrons; it is also evident that in 3 of the 4 cases the interval between the two exposures was 3 weeks. An additional experiment is being carried out, using this 3-week interval but a smaller neutron exposure (15 to 20 rads) as the "priming dose."

Figure 9 gives the 30-day mortality of all the age-control groups for both neutron and γ experiments. These probit lines indicate the earlier death following neutrons (60% dead by day 11) than following Co^{60} γ-rays (30% dead by day 11).

Mortality varied somewhat among the age-control groups, but in general a midlethal dose was achieved; every age control group showed some mortality after a dose of approximately $LD_{50/30}$ (323 rads neutrons or 900 rads Co^{60} γ-rays). If all the controls in the γ-ray experiments are combined (as in Figure 9), 100/188 or 53% died in the 30-day period following the exposure to Co^{60}. The comparable figure for the neutron age controls was 109 dead in the 30-day period out of a total of 218 irradiated or exactly an $LD_{50/30}$.

In order to determine whether the preliminary radiation affected over-all longevity, all mice were retained for the length of their lives. However, only in the 10-week groups were there adequate numbers of survivors of the acute period. In the 10-week groups a total of 60 mice were alive 100 days after the second exposure and in the age-control group 75 were alive.

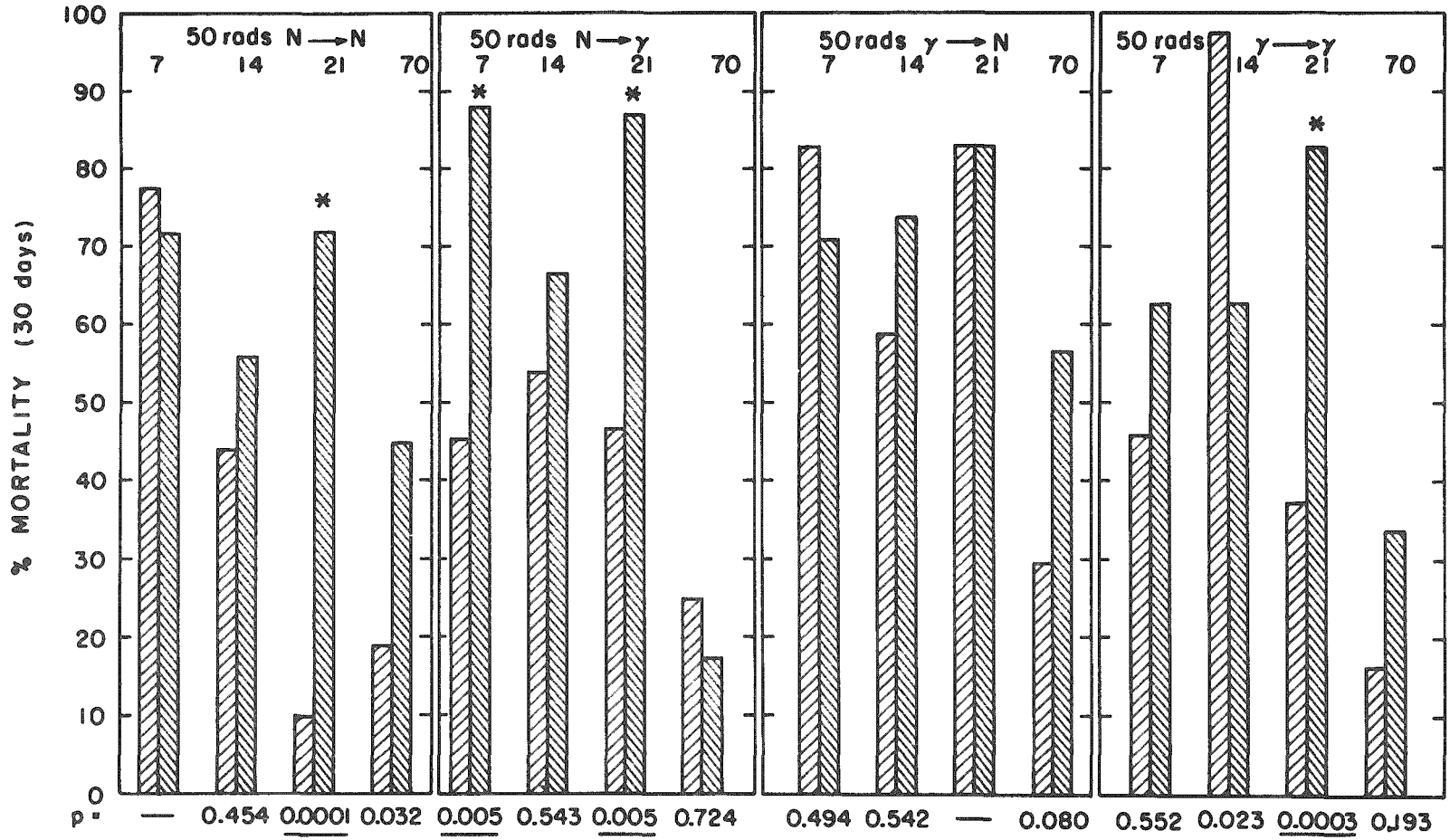

Figure 7. Data from 16 groups of mice given a preliminary dose of 50 rads of either fission neutrons or Co^{60} γ-rays, at 4 different intervals, prior to a second dose of either radiation. The 30-day mortality is illustrated for each group and compared with that of its age controls, which received only the second irradiation. Values beneath each set of bars are p values; the four significant figures (99% level) are underlined.

▨ Irradiated control mice; single mid-lethal dose of fission neutrons (320 rads) or Co^{60} γ-rays (900 rads).

▧ CF No. 1 ♀ mice irradiated with 50 rads (neutron or γ-rays) and given a second lethal exposure to the radiations 7, 14, 21, or 70 days after the first exposure.

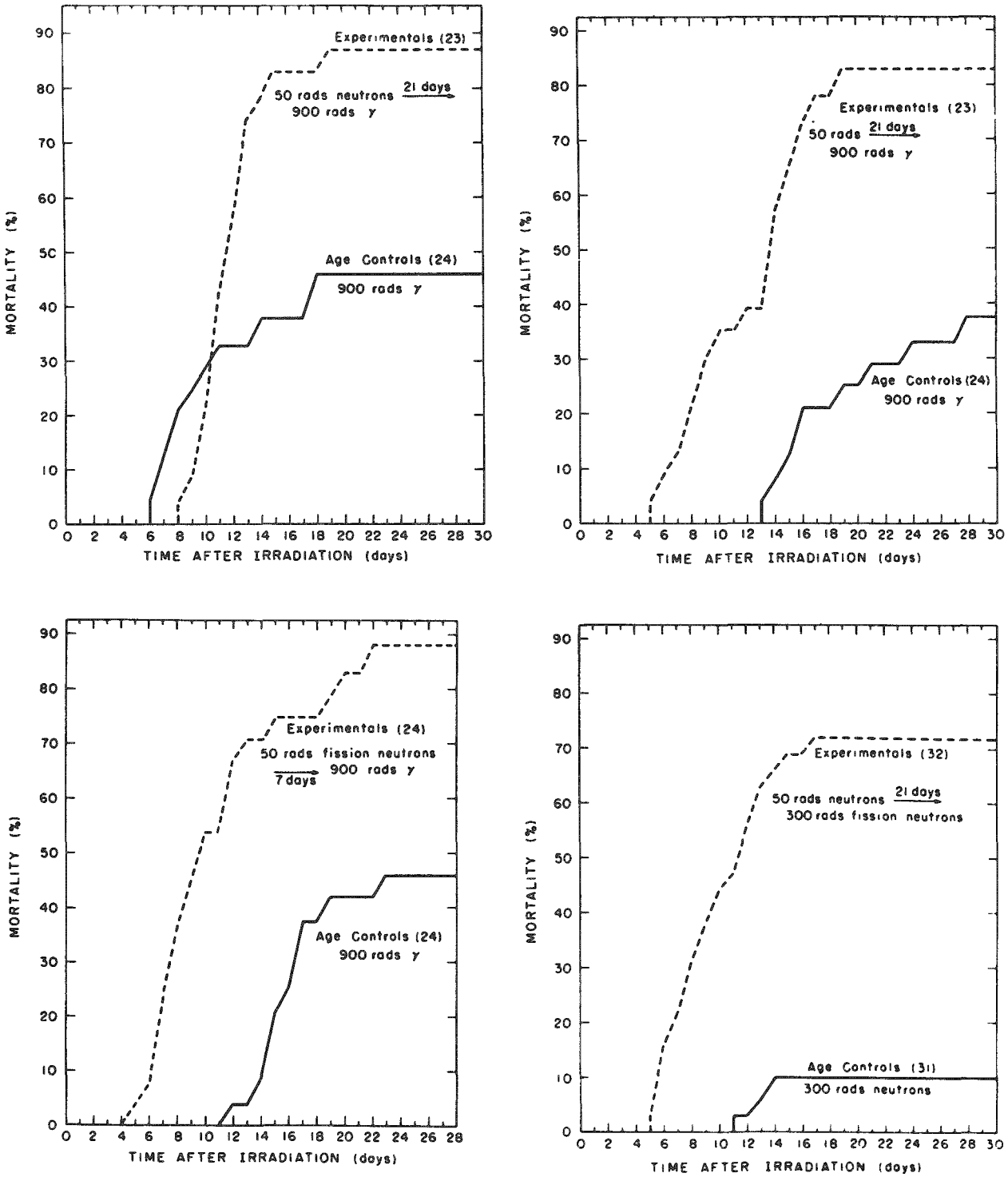

Figure 8. A comparison of the 30-day mortality data of the four experimental groups that showed significantly higher mortality than their age controls.

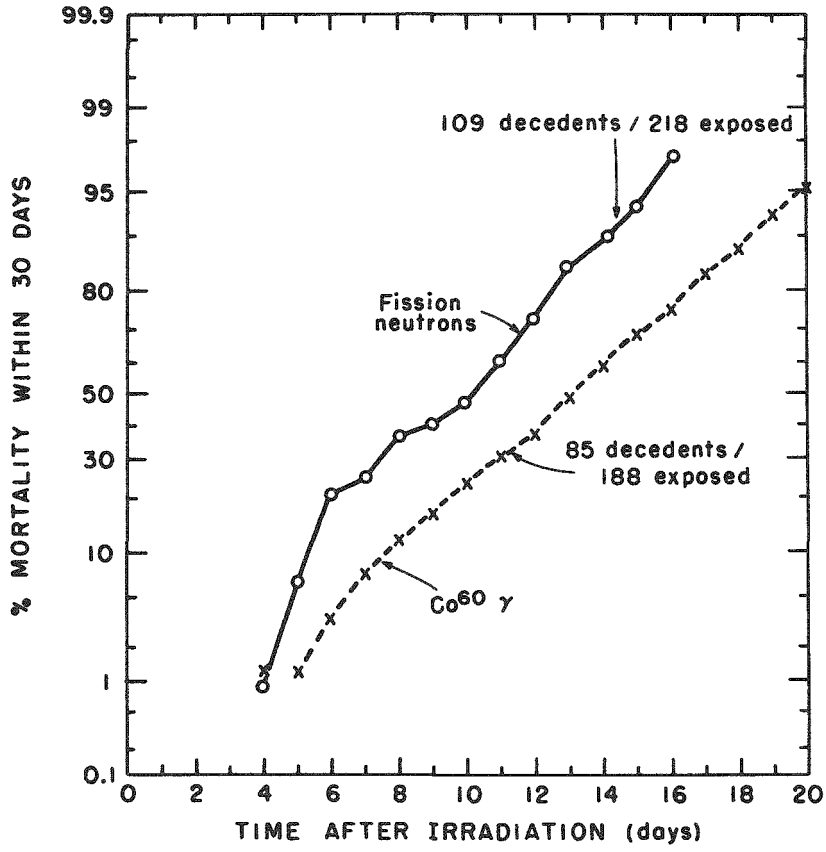

Figure 9. Thirty-day mortality of age controls for both neutron and γ-ray experiments.

Experimental and control animals are compared in Figure 10. It is clear that the mortality patterns of the two groups did not differ significantly, at least between 100 and 500 days after the second exposure. One minor difference was noted: 7 age-control mice died between days 30 and 100; none of the experimentals that survived the 30-day acute period died before day 100.

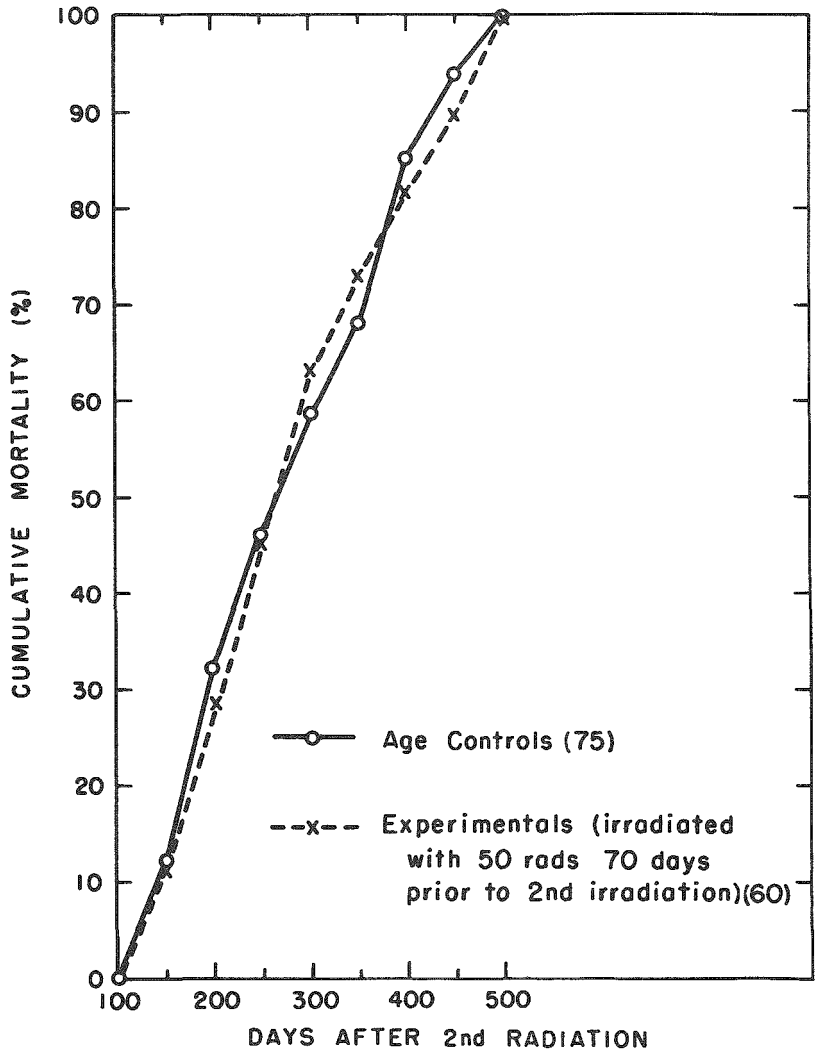

Figure 10. Cumulative mortality of the pooled 10-week interval groups and their pooled age controls. Values in parentheses represent numbers of animals surviving the 30-day acute period. The mean survival time of the controls was 276.6 days, that of the experimental animals, 274.7 days.

Reference

1. Dacquisto, M. P. Acquired radioresistance. A review of the literature and report of a confirmatory experiment. Radiation Research 10 118-129 (1959).

LONGEVITY OF FEMALE MICE IRRADIATED WITH SINGLE SUBLETHAL EXPOSURES OF FISSION NEUTRONS

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

The purpose of this report is to analyze longevity data from three separate experiments, all involving single sublethal irradiations of CF No. 1 female mice with fission neutrons.

Experiment I is represented by the 3 open circles of Figure 11. Three groups of 36 mice each were irradiated with a single whole-body exposure to neutrons at a dose rate of approximately 8 rads/min. The exposure times were brief, 10, 15, and 20 min respectively, to achieve single doses roughly equivalent to $\frac{1}{4}$, $\frac{1}{3}$, and $\frac{1}{2}$ of the $LD_{50/30}$ dose for these mice (323 rads). The three points suggest a linear relationship between the total dose and the average length of life of the irradiated mice: the higher the dose, the shorter the life span. Mean survival times and percent decrease in life span are shown in Table 7.

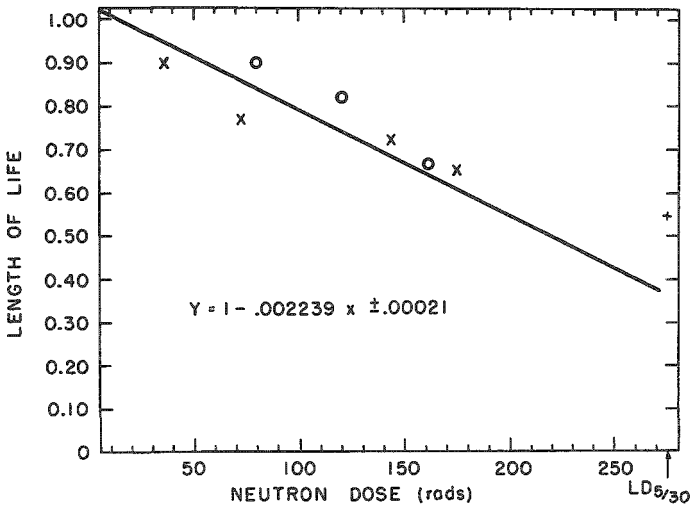

Figure 11

The effects of single sublethal irradiations with fission neutrons on length of life in CF No. 1 female mice.

In Experiment II, represented by the X's of Figure 11, the CP-5 reactor was operated at low power (50 kw rather than 2000 kw). The dose rate was thus decreased by a factor of approximately 40, and the mice were exposed to a neutron dose rate of approximately 12 rads/hour. As can be seen in Table 7, the irradiation times in this experiment varied from 3 to 27.5 hr. The highest dose proved to be acutely lethal; 44 of the 47 exposed mice died within 4 weeks. This group, therefore, is not represented in the figure. The results for the other 4 groups appear to confirm the relationship between neutron dose and survival time shown by the first experiment. It would, therefore, appear that the total dose of fission neutrons is more important than the intensity in reducing life span in these mice, at least within a factor of 40 as in these experimental conditions.

TABLE 7

Longevity in CF No. 1 female mice given single sublethal exposures to fission neutrons at dose rates varying by a factor of 50

Dose, rads	Exposure time, min	No. mice	Mean aftersurvival, days ± S. E.	Decrease in life span, %
		Experiment I		
Control	0	19	487.4 ± 35.1	
80	10	36	442.7 ± 23.3	9.2
120	15	36	398.6 ± 26.9	18.2
160	20	36	315.1 ± 23.1	35.4
		Experiment II		
Control	0	40	552.8 ± 20.1	
36	180	47	498.5 ± 15.9	9.8
72	360	48	425.7 ± 21.8	23.0
144	720	48	402.2 ± 23.6	27.3
174	870	48	362.7 ± 23.8	34.4
330	1650	47	21.9 ± 10.9	96.0
		Experiment III		
Control	0	29	477.6 ± 32.0	
275	48	36	262.1 ± 20.3	45.1

Experiment III is represented by the remaining point (+) in Figure 11, which gives the mean survival time of the 36 mice exposed to 275 rads of neutrons in a single, whole-body dose delivered in 48 minutes.[1] In this case the life span was decreased by 45% as compared to the control, unirradiated mice of this experiment.

A single regression line was then fitted to these eight points representing single doses of fission neutrons from 36 to 275 rads. The line illustrated in Figure 11, is represented by the equation $Y = 1 - 0.00224 X$. From these data it appears that a straight line with a slope of 0.22 represents the relationship between dose of fission neutrons and length of life. It can, therefore, be stated that, under the conditions of exposure at the CP-5 reactor, the life span of CF No. 1 female mice was shortened approximately 0.22% per rad of fission neutrons. This relationship between neutron dose and life shortening appears to be linear for neutrons, at least in the range of 10 to 85% of the $LD_{50/30}$ value.

Reference

1. Vogel, H. H., Jr., and D. L. Jordan. The effect upon mouse longevity of fractionation of a dose of fission neutrons. Biological and Medical Research Division Semiannual Report, Argonne National Laboratory. ANL-5916 (1958), pp. 95-96.

MAMMARY TUMOR INCIDENCE IN FEMALE SPRAGUE-DAWLEY RATS IRRADIATED WITH EITHER FISSION NEUTRONS OR Co^{60} γ-RAYS

Progress report (13 month period after first irradiation).

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

In this progress report we are comparing the incidence of mammary tumors in female Sprague-Dawley rats given four exposures at weekly intervals to either 100 rads of fission neutrons or 200 rads of Co^{60} γ-rays. The 57 neutron-irradiated rats were divided into four groups; each received the 100 rads at four different intensities (1, 3, 6, and 35 rads/min).(1) Similarly, the 55 rats exposed to Co^{60} γ-rays were divided into four dose-rate groups.

In Figure 12 are given the incidence of mammary tumors and the cumulative mortality following neutrons and γ-rays. All dose-rate groups are combined. Over the period of 13 months since the first irradiation, approximately 75% of the exposed rats developed at least one mammary tumor. In this same period only 2 of the 35 unirradiated controls showed any tumors and none died. It is clear from this figure that the neutron-irradiated rats (100 rads x 4) died much earlier than the γ-irradiated animals, even though the latter received twice the dose (200 rads x 4).

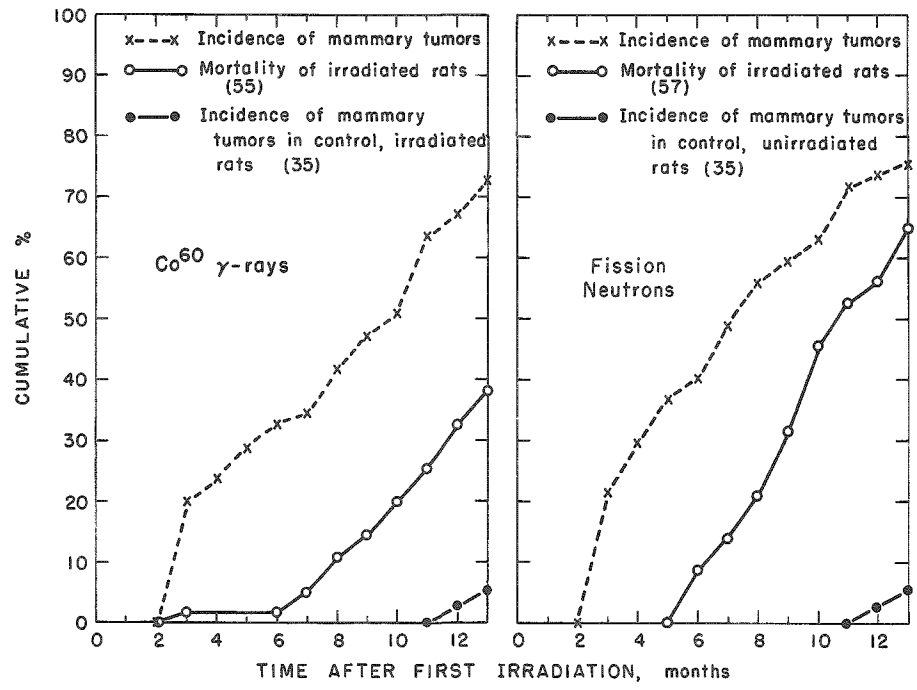

Figure 12. Cumulative percent incidence of mammary tumors in female Sprague-Dawley rats exposed to 4 weekly doses of fission neutrons or Co^{60} γ-rays. Numbers of animals are in parentheses.

For each radiation the slopes of the lines representing tumor incidence and mortality appear to be parallel. However, the time between the occurrence of tumors and the resultant mortality appears to be considerably shorter after neutrons (3-4 months) than after Co^{60} γ-rays (5-7 months).

Several of these rats developed multiple tumors before death: During the first 400 days after first exposure, 17 neutron-irradiated rats had 2 or 3 different mammary tumors, whereas only 5 γ-irradiated rats had more than 1 tumor.

The incidence of tumors in each dose-rate group is shown in Figure 13 for each radiation and the survival data for these same groups are illustrated in Figure 14. The effects of intensity will be analyzed when the experiment is completed.

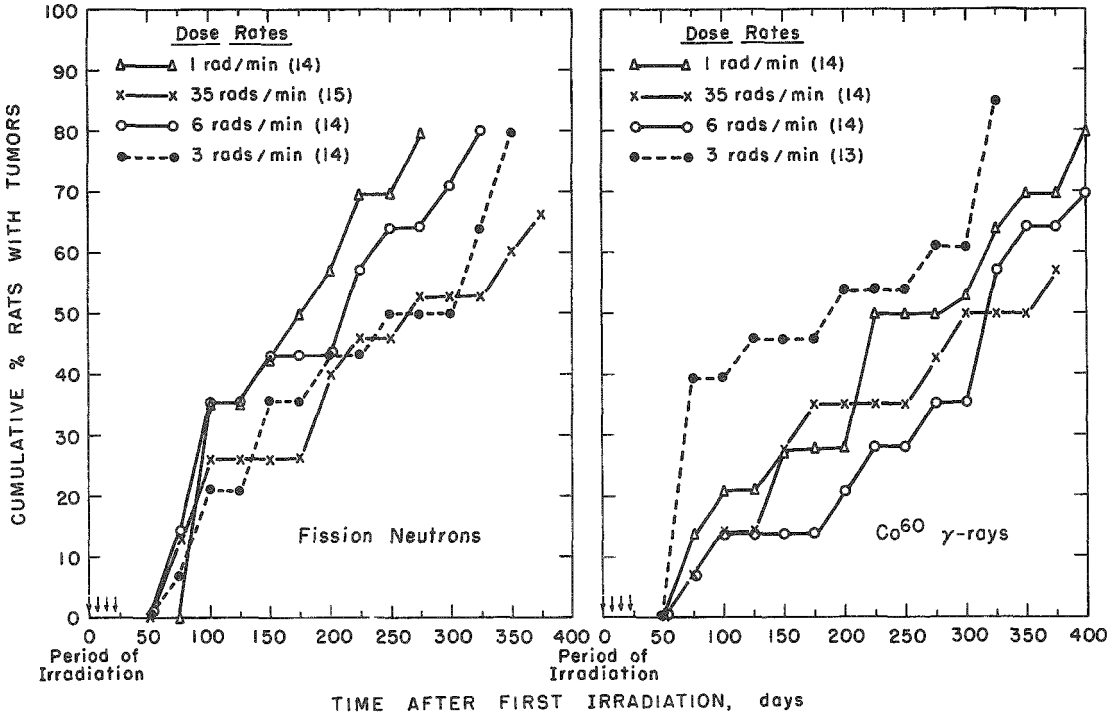

Figure 13. Cumulative percent incidence of mammary tumors in female Sprague-Dawley rats exposed to 4 weekly doses of fission neutrons or Co^{60} γ-rays at different dose-rates. Numbers of animals are in parentheses.

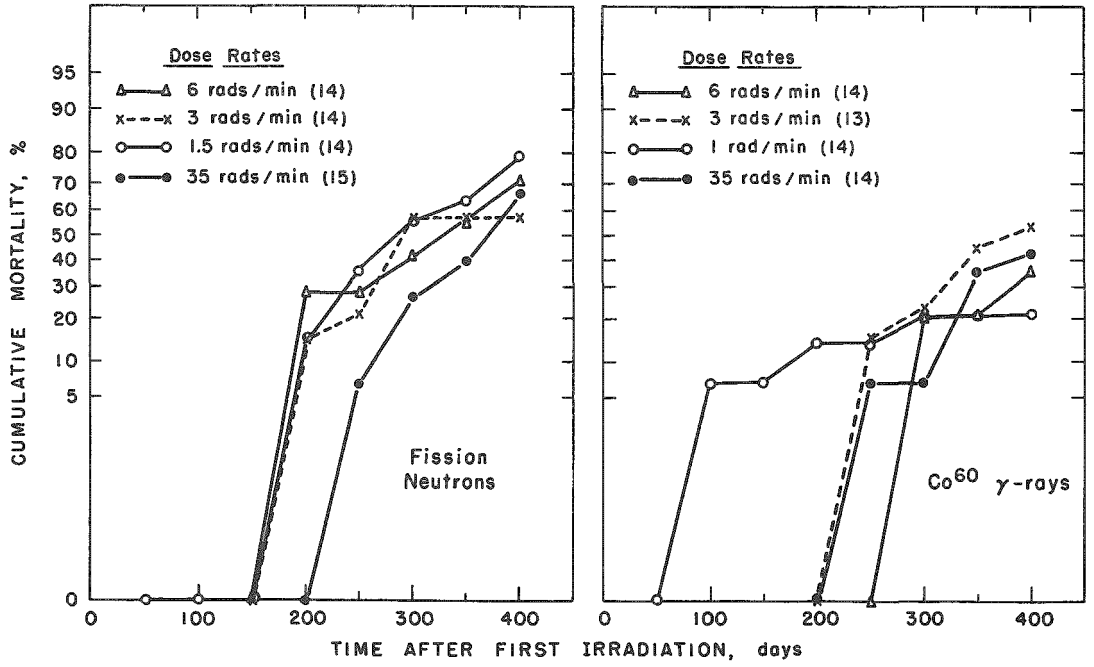

Figure 14. Longevity of female Sprague-Dawley rats given 4 weekly exposures to fission neutrons or Co^{60} γ-rays. Numbers of animals are in parentheses.

Reference

1. Vogel, H. H., Jr., and D. L. Jordan. Effect of neutron dose rate on the incidence of mammary tumors in female Sprague-Dawley rats. Argonne Biological and Medical Research Division Semiannual Report, Argonne National Laboratory. ANL-6264 (1959), pp. 29-30.

A FUNCTION FOR THE TISSUE MAST CELL

L. R. Draper* and D. E. Smith

The tissue mast cells have been shown to contain histamine, 5-hydroxytryptamine, heparin and hyaluronic acid, substances having significant physiological action.(1) Functions for the tissue mast cells have not been demonstrated clearly, however. Information concerning their function should be obtainable by determining whether phenomena conceivably controlled or influenced by the mast cells in normal tissues are altered in tissues made substantially free of mast cells. This note is concerned with such an approach, using alterations in the permeability of blood vessels that have been observed to follow antigen-antibody reactions in tissue.(2)

The serous tissues of the peritoneal cavity of the Sprague-Dawley rat weighing about 150 g were the objects of study. The mast cells of these tissues were destroyed by the intraperitoneal injection of 20 ml of distilled water. This treatment causes the immediate disruption of mast cells, the debris of which is phagocytized during the ensuing 3 to 5 days. Thereupon, the serous tissues of the peritoneal cavity are quite normal in appearance except that they lack mast cells.(3-4) The effects of antigen-antibody reactions on vascular permeability, as determined by the method outlined by Ovary,(5) in normal rats (having an intact mast cell population in the serous tissues of the peritoneal cavity) were compared with rats treated with distilled water 6 to 10 days previously. In these tests, a dilution of antiserum (rabbit anti-egg albumin) was injected intraperitoneally in volumes of 1 or 5 ml. (No estimates were made of the activity of the antiserum in terms of the mass of antibody nitrogen/ml.) This was followed 3 hr later by the intravenous injection of 10 mg of crystallized egg albumin in 1.0 or 1.5 ml of a 1% solution of Evans Blue dye in 0.9% NaCl. Fifteen to twenty minutes later the animals were killed by ether anesthesia, and the peritoneal tissues were examined for leakage of the dye into the perivascular spaces. Normal rabbit serum was used as a control for the antiserum.

The mesentery and parietal peritoneum were found to have a dark blue color in normal rats treated with rabbit anti-egg albumin serum and egg albumin, whereas the blue coloration was markedly attenuated or absent in rats previously treated with distilled water. No extravascular blue coloration was seen in these tissues in either normal or water-treated rats into which normal rabbit serum was injected instead of the antiserum. Substantially the same results were obtained when the skin was used as the site of the antigen-antibody reaction, the mast cells having been previously destroyed by the local intradermal injection of distilled water. Preliminary experiments suggest that results similar to, though less striking than those

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described above, may be found in rats in which mast cell destruction has been effected by the injection of anti-mast cell serum prepared in rabbits[6] or by repeated administration of the histamine liberator, 48/80.

We interpret the findings of the present experiments to indicate that the increased permeability accompanying the antigen-antibody reaction depends, in part at least, upon the presence of the tissue mast cell, the release of its histamine and/or 5-hydroxytryptamine being directly responsible for the alteration in permeability. The mechanism by which the release of these substances is brought about is not clear. It is known from previous experiments, however, that mast cells disrupt and release their granules into the surrounding tissue in passive anaphylaxis[7] and recently, Archer[8] has reported that a heat-labile substance is formed during an antigen-antibody reaction which causes the disruption of rat mast cells in vitro. That mast cells may initiate inflammatory processes following other tissue injury in rats is suggested by Sheldon and Bauer.[9] The present results indicate that the mast cells may be of primary importance in initiating the inflammatory response accompanying antigen-antibody reactions.

References

1. West, G. B. Tissue mast cells and tissue amines. J. Pharm. Pharmacol. 11 513-534 (1959).
2. Fischer, J. P. and R. A. Cooke. Passive cutaneous anaphylaxis (PCA) in one guinea pig. J. Allergy 28 150-169 (1957).
3. Fawcett, D. W. An experimental study of mast cell degeneration and regeneration. Anat. Rec. 121 29-51 (1955).
4. Smith, D. E. and Y. S. Lewis. Phagocytosis of granules from disrupted mast cells. Anat. Rec. 132 93-112 (1958).
5. Ovary, Z. Cutaneous anaphylaxis in the albino rat. Intern. Arch. Allergy and Appl. Immunol. 3 203-301 (1952).
6. Smith, D. E. and Y. S. Lewis. Preparation and effects of an anti-mast cell serum. J. Exptl. Med. 113 683-692 (1961).
7. Humphrey, J. H. and I. Mota. The mechanism of anaphylaxis: Specificity of antigen-induced mast cell damage in anaphylaxis in the guinea pig. Immunol. 2 31-43 (1959).
8. Archer, G. T. Release of histamine from mast cells by antigen-antibody reactions. Austral. J. Exptl. Biol. 38 147-152 (1960).
9. Sheldon, W. H. and H. Bauer. Tissue mast cells and acute inflammation in experimental cutaneous mucormycosis of normal, 48/80-treated, and diabetic rats. J. Exptl. Med. 112 1069-1083 (1960).

PROGRESS REPORT: DISEASES AND CARE OF LABORATORY ANIMALS

I. Effects of Pseudomonas Infection of Mice

R. J. Flynn, E. J. Ainsworth and I. Greco

Pseudomonas infection interferes with radiobiological studies and probably with many other stress studies as well. In the presence of such infection the results of postirradiation protection experiments involving therapeutic agents such as bone marrow and bacterial pyrogens cannot be satisfactorily explained because if the lethally irradiated animals are infected, they die before any therapy can be effective. Because many radiological laboratories[1-4] as well as our own have been affected by this problem, an effort is being made to resolve it.

Effects on Radiobiological Studies. The effects of Pseudomonas infection on survival time after exposure to a lethal dose of X-rays is graphically illustrated in Figure 15. Before the colony became infected, the mean survival time of CF No. 1 (Argonne) "pathogen-free" mice exposed to 700 r was 13.81 days. When infected mice were exposed to irradiation, their mean survival time dropped to 6.01 days.

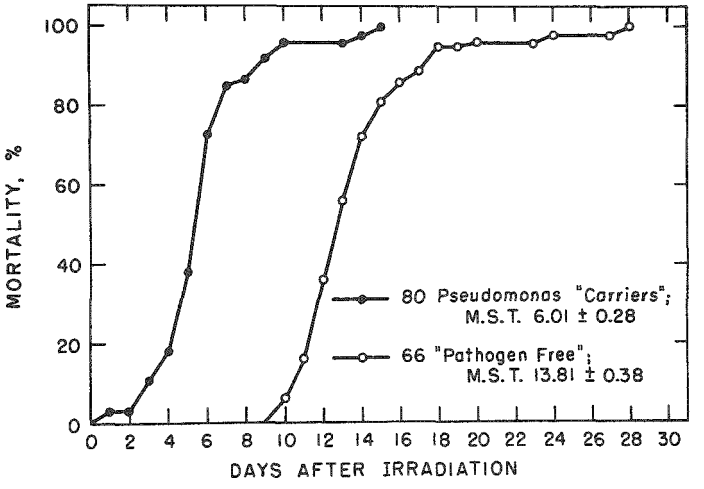

Figure 15

The effects of Pseudomonas infection on the mean survival time (MST) of mice after exposure to a lethal dose of X-rays.

The survival pattern observed when some but not all of the irradiated animals are Pseudomonas carriers, the usual situation, is illustrated in Figure 16. Note the slight but definite bimodality and the "shift to the left" (decreased survival time). Also shown in this figure is the survival curve for similarly irradiated mice free of Pseudomonas.

A differential radiation sensitivity, in terms of 30-day survival, exists between Pseudomonas carriers and Pseudomonas-free mice, but the current data are insufficient to quantitate this difference. It has been

observed repeatedly, however, that the $LD_{99\ 30}$ for Pseudomonas-free CF No. 1 (Carworth) mice is approximately 750 r, whereas 650 r produces comparable mortality in Pseudomonas-infected CF No. 1 (Argonne) mice. (also see below).

Attempts were made to reproduce the early death effect illustrated in Figure 15 by feeding Pseudomonas in drinking water to noninfected mice. With the establishment of an efficient "gut-seeding system," attempts could be made to alter the gut flora experimentally (with Lactobacillus, for example) in such a way as to render the mice more resistant to natural contamination with Pseudomonas organisms.

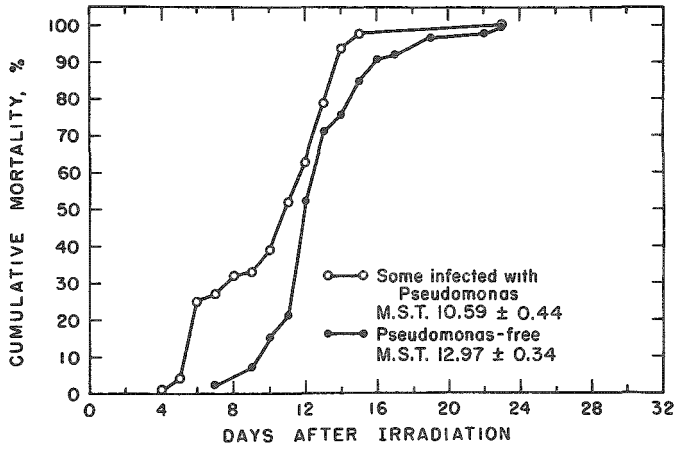

Figure 16

The survival pattern of lethally irradiated (900-1200 rads Co^{60} γ rays) mice when only some of the animals are Pseudomonas carriers. Also shown is the survival pattern for mice free of Pseudomonas.

In a preliminary experiment, Pseudomonas organisms (6.5×10^8/ml) were fed to uninfected CF No. 1 (Argonne) mice for 24 hr 7 days before irradiation, and in this trial the typical survival curve of irradiated infected mice resulted. A challenge dose of 6.5×10^7 organisms did not produce this effect, thus indicating some sort of threshold. When another group of 40 uninfected CF No. 1 (Argonne) mice was fed Pseudomonas 14 days before being given 700 r, cultures of the heart's blood and guts of all decedents and 30-day survivors revealed that only 6 of the 40 mice became infected. No early deaths occurred, and 25 of the mice survived ($LD_{37.5/30}$). These results indicate that production of early death by a single feeding of Pseudomonas is influenced both by the time of feeding and by the challenge dose of organisms. No attempt was made to determine the number of Pseudomonas organisms that became "implanted" in the intestine. A great attrition undoubtedly occurs in the stomach, and the results of feeding 14 days before irradiation suggest that most of the organisms that reach the gut are eliminated.

When another group of 39 noninfected CF No. 1 (Argonne) mice was inoculated 4 days before irradiation, and the water bottles were refilled but not exchanged during the entire experiment, the results were dramatically different. Pseudomonas organisms were recovered from the feces or heart's blood of 35 of the 39 mice, many early deaths occurred, and only 5 of the 39 mice survived ($LD_{87.2/30}$).

Effects on Other Types of Research. It has been suggested[5] that the observed decreased survival time of CF No. 1 (Argonne) mice following inoculation with EO(H) ascites tumor may be due to Pseudomonas infection. It has also been reported that Pseudomonas infection interferes with burn therapy studies.[6] The stress associated with the burns apparently activates ths Pseudomonas infection in a manner similar to that of irradiation, i.e. the mean survival time is decreased and the mortality increased.

Pseudomonas-infected mice also appear to be less resistant to experimental infections. An illustration of this is given in Table 8. Infected CF No. 1 (Carworth) mice were challenged with intraperitoneal injections of saline suspensions of living Proteus morganii. The Pseudomonas-infected substrain was far more susceptible to the bacterial challenge than was the uninfected substrain. However, it was not clearly shown that this effect was entirely attributable to the Pseudomonas "carrier state" and not to some other environmental or inherited difference.

Table 8

Intraperitoneal challenge with living Proteus morganii

Challenge dose, number of organisms	72-hr mortality	
	CF No. 1 (Carworth) uninfected	CF No. 1 (Argonne) infected
15×10^7	10/25	14/15
10×10^7	0/25	12/15
5×10^7	0/25	3/15
2.5×10^7	0/25	0/15

II. Studies on the Diagnosis of Pseudomonas Infection of Mice

R. J. Flynn and I. Greco

The fact that Pseudomonas infection interferes with radiobiological research makes it eminently desirable to eliminate the organisms from the experimental animals. Therefore, a rapid, accurate method of diagnosis, preferably in the living animal, is an essential prerequisite. The present report describes 1) attempts to detect Pseudomonas organisms by means of differential growth characteristics on several bacteriological culture media, and 2) results of studies of the incidence of recovery of this organism from water bottles known to be contaminated.

Comparative Studies of Culture Media and Incubation Temperatures. Pure cultures of 8 pigment-producing and 3 supposedly non-pigment-producing strains of Pseudomonas aeruginosa were used in these studies. For comparative purposes, typical strains of other enteric bacteria were also used. These were Salmonella typhimurium, Alkaligenes faecalis, Streptococcus faecalis, Escherichia coli, Paracolobactrum sp., Aerobacter aerogenes, Proteus morganii, and three cultures of Proteus mirabilis. Thirteen different media were used: tetrathionate broth, triple sugar iron agar slants, brain-heart infusion broth, lead acetate agar slants, lactose broth, Simmons citrate agar slants, glycerol broth,[1] TGY (tryptone, glucose, yeast) agar slants, SS (Salmonella-Shigella) agar plates, EMB (eosin methylene blue) agar plates, blood agar plates, glycerol agar plates,[1] and gelatin. The gelatin was incubated at 26°C; the other media at 37°C and 41°C. Observations were made for growth, hemolysis, pigment production, and any other morphological or biochemical characteristics that might aid in the differential diagnosis of Pseudomonas infection.

At 37°C, most of the organisms grew well on most of the media. Exceptions were the two species of Proteus, which failed to grow on Simmons citrate agar, and S. faecalis, which failed to grow well on any of the media.

At 41°C, most of the organisms grew poorly. Exceptions were the eleven strains of P. aeruginosa, all of which grew well on triple sugar iron agar, blood agar, and glycerol agar. Another important exception was S. typhimurium, which not only grew very well on these media, but on most of the others also. Although this observation may be of little value in the differential diagnosis of Pseudomonas infection, it may be of great importance in the differential diagnosis of Salmonellosis and will be studied further. After 48 hr, all eleven strains of P. aeruginosa grew well on Simmons citrate agar at this temperature. Only A. aerogenes and, to a lesser extent, A. faecalis grew similarly.

Hemolysis of blood agar was produced at 37°C by S. faecalis (alpha hemolysis) and by 9 of the 11 strains of P. aeruginosa (beta hemolysis). At 41°C, only 5 of the 11 strains of P. aeruginosa were capable of producing hemolysis.

Blue-green pigment (pyocyanin) production was associated only with P. aeruginosa. At 37°C, of the 11 strains, 4 produced pigment in tetrathionate broth, 5 in brain-heart infusion broth, 8 on SS agar, 9 in glycerol broth*, and 10 on glycerol agar. (The one strain that failed to grow and produce blue-green pigment on glycerol agar was observed to have produced pigment on this medium on a previous occasion.) At 41°C, blue-green pigment was produced in glycerol broth by 4 of 11 strains, and on glycerol agar by 9 of the 11 strains. No other medium supported pigment production at this temperature.

* The vigorous shaking of glycerol broth tubes before reading greatly increases the intensity of the blue-green pigment, if present.

Gelatin was liquefied by one strain of P. mirabilis, and by all 11 strains of P. aeruginosa. (Serratia marcescens and most strains of Staphylococcus are also capable of producing this effect.)

A silvery metallic iridescence was observed associated with some of the strains of P. aeruginosa on certain media, but this was not a consistent finding. It was observed incidentally that the greenish metallic sheen produced on EMB agar at 37°C by E. Coli and occasionally by A. aerogenes was produced in profusion by the latter organism at 41°C.

An unusual precipitate, apparently crystalline, was noted to occur on SS agar after 72 hours' incubation at 37°C. Because this as yet unexplained finding was found associated with only S. typhimurium and 10 of the 11 strains of P. aeruginosa, it may have differential diagnostic value.

Although further studies of this latter phenomenon are indicated, the other results show that the one easily discernible characteristic of P. aeruginosa, namely the capacity to grow at 41°C,[7] is sufficiently ubiquitous among enteric bacteria to be of little or no diagnostic value. For the present at least, the practical diagnosis of Pseudomonas infection, therefore, must depend upon the detection of pigment production, and for this, glycerol broth and glycerol agar are the most sensitive.

Water-bottle Culturing. Glycerol broth and glycerol agar were used to test water bottles from 64 known infected cages of mice for P. aeruginosa in a variety of ways to determine the simplest and most efficient testing procedure.

Table 9 shows the comparative efficiency of incubating inoculated glycerol broth tubes for 12 days at 37°C and at 26°C. The higher incubation temperature is the most efficient. Also, positive reactions, as indicated by pigment production, occur earlier at the higher temperature.

Table 9

The influence of the time water bottles remained on cages before testing and of the incubation temperature on the efficiency of detecting Pseudomonas infection

Incubation temperature, °C	% Incidence of detection			
	Days on cages			
	1	2	3	4
37	82.9	93.7	98.5	96.9
26	79.7	79.7	89.1	92.2

Table 9 also shows that the accuracy (i.e. incidence of detection) of this method is related, for 4 days at least, to the length of time the water bottles are left on the cages before testing.

The efficiency of detection was influenced by the time of subculturing from glycerol broth to glycerol agar (Figure 17). Subculturing at day 1 was found to be more efficient than at day 3, and at day 3, more than at day 7. A comparison with the curves for broth culture alone shows, however, that subculturing from broth tubes to agar plates gave no more accurate results than using the broth tubes alone. (It should be noted that this observation applies only to the culturing of water bottles. When culturing feces and similar opaque or pigmented materials, the diagnostic blue-green pigment is not always easily discernible, and subculturing to agar is frequently necessary.)

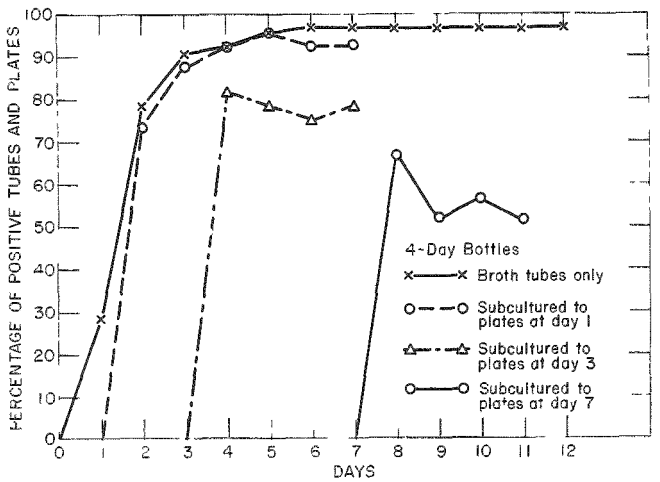

Figure 17

Influence of time of subculture on efficiency of detection. The 4-day water bottles were cultured on glycerol broth and subcultured on glycerol agar on days 1, 3, and 7.

III. Studies on the Epidemiology of Pseudomonas Infection in Mice

R. J. Flynn, I. Greco, and L. O. Bibbs

Before an infectious disease can be controlled, it is necessary to know its epidemiology, i.e., its method of spreading. Therefore, the possible vectors of Pseudomonas infection were studied.

Possible Inanimate Vectors. A number of inanimate materials (Table 10) were checked for P. aeruginosa, using glycerol broth and glycerol agar plates.

Of the samples listed, only two were positive: an air sample (60-minute plate count) from a rabbit room and an air sample (60-minute plate count) from a chicken room. In the former, of 110 colonies counted, only 2 were Pseudomonas colonies. In the latter, of 500 colonies counted, 2 were Pseudomonas. None of the other materials tested was found to be contaminated with the organism.

TABLE 10

Inanimate materials tested for Pseudomonas

Materials	No. of samples	Materials	No. of samples
Animal Bldg.		Animal Feeds	
Sinks	27	Mouse (Wayne)	78
Floors	25	Mouse (Staley-Rockland)	10
Door knobs	22	Mouse (Purina)	29
Walls	3	Mouse (Dietrich and Gambrill)	2
Cage wash. mach.	2	Rabbit (Wayne)	10
Injection hood	1	Guinea pig (Staley-Rockland)	13
		Dog (Friskies)	9
Animal Equip.		Chicken (Wayne)	14
		Monkey (Purina)	2
Water bottles*	96	Baby (Pablum)	1
Stoppers*	96	Dried milk	2
Nipples*	96	Bird seed	2
Rodent cages*	100	Alfalfa hay	1
Chicken cages	4	Lettuce	1
Racks	7	Sugar	1
Scale pans	1	Wheat germ oil	1
		Vit. A and D oil	1
Air Samples		Gravel	1
Rodent rooms	19		
Rabbit rooms	3	Water	
Chicken rooms	3		
Dog room	1	Drinking water	96
Kennels	2		
Grasshopper room	1	Bedding	
Cage wash. room	2		
Food storeroom	1	Wood shavings	100
Surgery room	1		
Injection room	1	Vaccine	
		Mouse pox	21

*After sanitizing

Possible Animate Vectors. The hands of 12 animal technicians were examined as they arrived at work and again at the end of the work day. Eleven were consistently negative, both on arriving and leaving; one was consistently positive. Even after repeated scrubbing and a 10-day absence, the hands of this one individual remained infected.

Another animate vector that may be of equal or greater importance is the cockroach. Of 62 cockroaches mashed and cultured in glycerol broth, 17 were found to be harboring P. aeruginosa. To determine whether the cockroach is merely a mechanical vector or if it carries the infection internally, as does the grasshopper,[8] 97 additional cockroaches were cultured but not mashed. All 97 were negative.

Additional Animate Vectors. Obviously the infected mice themselves are a possible source of contamination to their cagemates, and presumably to all other mice in the same room. Although we are still in the process of acquiring detailed data concerning the frequency and periodicity of passage of P. aeruginosa in the feces of infected animals, our data to date indicate that infected mice do pass the organism most of the time. Since it has been shown that mice can be infected by the oral feeding of Pseudomonas (Section I), the spread of infection within a cage seems, therefore, to be simple and direct

The spread of infection from mother to young is also apparently direct, thus perpetuating infection within a colony.

But the spread of infection from infected cages to unrelated, uninfected cages was deduced only after it was observed that uninfected animals moved into an infected breeding room, but separately caged, remained free of infection, while at the same time, an infection that had gained entrance into a formerly uninfected breeding room spread rapidly. When it was noted that the infection in the latter room was spreading horizontally and downwardly within a rack, without regard for familial relationships, the method of spread from cage to cage became apparent.

In the room in which the infection failed to spread, the breeding stocks were housed in steel cages with solid sides, wire bottoms, a dropping pan, and without bedding. In the room in which the infection was spreading, the stocks were housed in plastic cages with perforated metal tops and with wood shavings as bedding. Mice housed in these latter cages, especially during the nocturnal periods of high activity, threw soiled bedding and feces out through the perforations in the cage top onto the rack shelf and floor, and onto or into any closely adjoining cages. Cultures made of such soiled bedding taken from the tops of cages, from between cages, and from the floor in front of the infected rack were found to be infected with Pseudomonas.

IV. Disease-free (Pathogen-free) Breeding Colony

R. J. Flynn and L. O. Bibbs

For the last few years, efforts have been made to maintain a disease-free rodent breeding colony within the existing animal quarters. This has been difficult, first, because the facilities available have been inadequate, and, second, because we lack knowledge of the epidemiology of many of the common diseases of laboratory rodents.

The first attempt at maintaining such a colony failed because the animals became infected with the oxyurids (pinworms) Syphacia obvelata and Aspicularis tetraptera. Efforts to determine the source of these infections were to no avail, and one could only assume that an undetected error in technique had probably occurred.

The next attempt failed because the mice became heavily infected with Pseudomonas. When it was learned that the caretaker responsible for these animals was a Pseudomonas carrier (Section III), a personnel change was made and a new colony was initiated.

Concurrent with the development of these colonies, studies were started concerning the diagnosis and epidemiology of the common diseases of laboratory animals, particularly Pseudomonas infection (Sections II and III). Therefore, when the third colony became infected on three separate occasions, the infection was discovered promptly and quickly eliminated, usually by destroying only a very small percentage of the total population.

Unfortunately, the sources of these outbreaks were not conclusively proven. One appeared to result from the heavy infestation of the entire animal quarters with cockroaches, now known to be a source of infection (Section III). Improvements in the vermin control program have reduced this problem to a minimum but have not eliminated it.

In another outbreak, the diet was suspected. All attempts to isolate Pseudomonas from the nonsterilized food were unsuccessful. However, the fact that only one infected food pellet may be enough to destroy the value of the entire colony suggested the use of a sterilized diet. Although a supposedly pathogen-free pasteurized diet is currently available commercially, an investigation of the method employed in processing this food indicates that while Pseudomonas contamination is unlikely, it is not completely eliminated. Therefore, a commercially available mouse diet that is supposedly sterilizable at 15 pounds steam pressure for 25 min, while remaining nutritionally adequate, is currently being tested.

The source of the third outbreak has not been determined and could be due to any of the previously suggested possibilities. Thus, although progress has been made, much is still to be learned.

However, concerning the positive achievements of this program, it must be noted that the last two colonies have been entirely free of all endoparasites, and that all colonies have been free of ectoparasites and such other commonly occurring diseases as salmonellosis.

The long-range objective of this program is to produce uniformly healthy research animals. Resistance to radiation in itself is not indicative of uniformity or good health. However, because radiosensitivity is frequently indicative of a latent infection or poor health, the fact that mice produced in these colonies (when not infected with Pseudomonas) have proved to be relatively radioresistant can only be encouraging. In a small trial involving 40 100- to 110-day-old mice, the $LD_{50/30}$ was found to be in excess of 700 r. In another trial involving 150 female CF No. 1 (Carworth) mice (7 to 9 weeks old) versus 150 female CF No. 1 (Argonne) mice (7 to 8 weeks old), the $LD_{50/30}$ for the Carworth mice was found to be below 500 r, while that of the Argonne mice was in excess of 650 r.

References

1. Wensinck, F., D. W. Van Bekkum, and H. Renaud. The prevention of Pseudomonas aeruginosa infections in irradiated mice and rats. Radiation Research 7 491-499 (1957).

2. Upton, A. C. Personal communication (1961).

3. Simmons, E. L. Personal communications (1961).

4. Plzak, V. and J. Doull. Pharamacological and toxicological compounds as protective or therapeutic agents against radiation injury in experimental animals. II. The influence of various chemical compounds on radiation lethality in mice. The University of Chicago USAF Radiation Laboratory Quarterly Progress Report No. 38. NP-9857 (1961) pp. 75-77.

5. Flynn, R. J Pseudomonas infection of mice. Biological and Medical Research Division Semiannual Report, Argonne National Laboratory. ANL-6264 (1959) pp. 155-157.

6. Millican, R. C. Personal communication (1961).

7. Haynes, W. C. Pseudomonas aeruginosa - its characterization and identification. J. Gen. Microbiol. 5 939-950 (1951).

8. Bucher, G. E., and J. M. Stephens. A disease of grasshoppers caused by the bacterium Pseudomonas aeruginosa (Schroeter) Migula. Can. J. Microbiol. 3 611-625 (1957).

THE IMMUNE RESPONSE IN HIBERNATING ANIMALS

I. Antigen Disappearance in Hibernating Ground Squirrels (Citellus tridecemlineatus)

B. N. Jaroslow and D. E. Smith

The resistance of hibernating animals to infection has been studied by a number of workers.[1] It would appear that increased resistance to infection is concomitant with entry into hibernation. There is no evidence to suggest that increased resistance to infection is other than a nonspecific effect associated with physiological changes accompanying this process. To our knowledge, the immune response has not been studied in mammals during hibernation. The present study is concerned with antigen disappearance in the hibernating ground squirrel (Citellus tridecemlineatus).

The ground squirrels were collected in northern Illinois during September 1960 and were maintained in a room at 23°C for six weeks prior to use. During this period they were individually caged and allowed free access to Rockland guinea pig diet with supplements of carrots twice weekly. Hibernation was induced by placing the animals, individually caged in a deep bed of wood shavings without food or water, in a room at 5°C and 50% relative humidity. They were checked twice daily to determine their state of hibernation.

We followed the disappearance of I^{131}-labeled bovine serum albumin (BSA) from the circulation of hibernating and nonhibernating ground squirrels. All squirrels received a single intraperitoneal injection of 10 mg of BSA labeled with I^{131} according to the method of Talmage et al.[2] Serum from ground squirrels was iodinated and injected by the same procedure.

Blood samples from the tail were collected on tared filter paper, weighed, and counted in a well-type scintillation counter. Counts were corrected for background, disintegration and weight of sample, converted to percentage of the activity of the sample taken one day after antigen injection, and plotted on a semilogarithmic scale. The rate of antigen disappearance was in three phases, as indicated by marked changes in the slope of the plot. These three phases were, in the terminology of Dixon et al.,[3] first, the equilibration phase, second, the nonimmune elimination phase, and third, the immune disappearance phase which marks the appearance of antibody.

In Figure 18 the mean slopes are plotted for each phase of antigen disappearance in the 4 treatment groups. Thus, the mean slope for the period of equilibration is an average of the slopes of that phase for the individuals in the group. The mean slopes for the nonimmune elimination and the immune phases are obtained in the same manner.

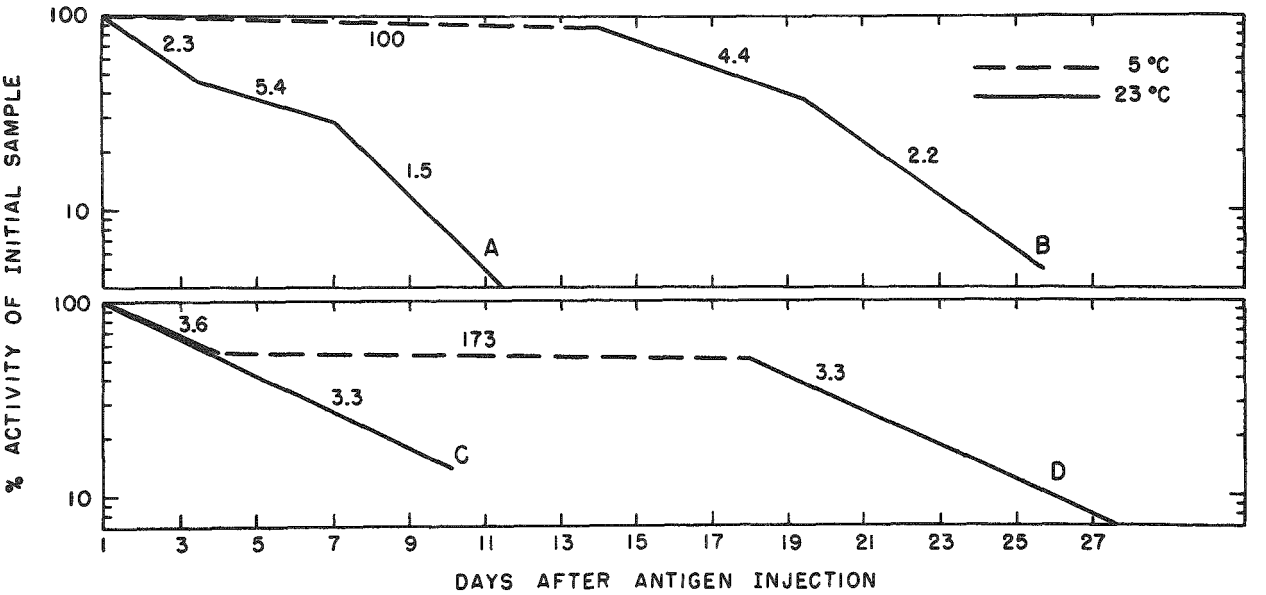

Figure 18. A semilog plot of the mean disappearance curves of I^{131}-labeled BSA in groups A and B, and of I^{131}-labeled ground squirrel serum in groups C and D. After injection of labeled material on day 0: A, 14 animals maintained at 23°C; B, 11 animals maintained at 5°C for 14 days in hibernation followed by a return to 23°C; C, 4 animals maintained at 23°C; D, 5 animals at 23° C for 4 days, then in hibernation at 5°C for 14 days followed by a return to 23°C. The numbers associated with each phase of the plot show the half-disappearance rate in days.

Group A is composed of 14 squirrels that remained at room temperature after injection of antigen. Blood samples were taken 5 times during the first 7 days and 3 times during the next 5 days. The period of equilibration in the animal lasts as long as 3.5 days with a half-disappearance time of 2.3 days. During the nonimmune elimination phase, which follows, the rate of antigen disappearance slows so that the antigen level decreases by half in 5.4 days. On the seventh day after antigen injection the half-disappearance rate becomes 1.5 days. This increase in rate of disappearance is considered to mark the end of the induction period and the beginning of the immune phase wherein the appearanc of antibody in the circulation is followed by rapid removal of the circulating antigen-antibody complexes. This sequence is qualitatively similar to that observed by Dixon et al.[3] in rabbits.

Group B is composed of 11 animals that were placed in the cold room immediately after antigen injection. They all entered hibernation within a day. During the 2-week hibernation period only 4 samples were taken; following arousal the sampling schedule was the same as for group A. There was little or no disappearance of antigen during the period of hibernation. Upon arousal, the half-disappearance rate became 4.4 days, which was not statistically different from that of the nonimmune elimination phase in A (5.4 days). After 5 days the end of the induction period was marked by a change in the half-disappearance rate to 2.2 days.

To determine whether the rate of disappearance of the antigen upon awakening from hibernation was comparable to the nonimmune disappearance rate or was actually the result of physiological processes peculiar to arousal, we studied the disappearance rate of labeled squirrel serum.

In 4 squirrels (group C) injected with labeled serum the half-disappearance rate was 3.3 days. In 5 similarly treated squirrels (group D) that entered hibernation 4 days after serum injection, the half-disappearance rate upon arousal from 14 days of hibernation was 3.3 days. This was almost identical with the half-disappearance rate of 3.6 days for the same animals during the 4 days prior to hibernation and for the group C animals that did not hibernate. In group D, as in group B, there was little or no disappearance of the labeled material during hibernation. Therefore, it is safe to assume that the disappearance rate of antigen in squirrels coming out of hibernation is the reflection of a metabolic state (measured by antigen disappearance) comparable with that of the nonhibernating ground squirrels.

Although there is little or no disappearance of homologous or heterologous proteins from the circulation of hibernating ground squirrels, the induction period ends 5 days after arousal (group B, Figure 18). Since the normal induction period is 7 days (group A, Figure 18), it would appear that some of the events that occur during the induction period transpired while the ground squirrels were hibernating. Work is in progress to determine whether the whole or only part of the induction period can be passed in hibernation.

We wish to thank Miss Joan A. Stachura for her technical assistance.

References

1. Kalabukhov, N. I. Characteristics of heat regulation in rodents as one of the factors in their sensitivity to plague infection. J. Microbiol. Epidemiol. Immunobiol. (U.S.S.R.) 29 1453-1460 (1958).

2. Talmage, D. W., H. R. Baker, and W. Akeson. The separation and analysis of labelled antibodies. J. Infectious Diseases 94 199-212 (1954).

3. Dixon, F. J., S. C. Bukantz, and G. J. Dammin. The effect of sensitization and X-radiation on the metabolism of I^{131} labeled proteins. Science 113 274-276 (1951).

THE DNA SYNTHESIS TIME OF THE MYELOCYTE IN THE DOG

M. A. Maloney, C. L. Weber and H. M. Patt

This communication is concerned with the DNA synthesis time of the myelocyte as revealed by autoradiographic analyses of bone marrow after administration of tritiated thymidine. A single injection of tritiated thymidine can be thought of as a flash labeling; and the initial labeling index is approximately proportional to the percentage of cells in DNA synthesis. About 20% of myelocytes are labeled within 30 min after thymidine injection. A DNA synthesis time for the dog myelocyte of about 5 hr was inferred from our previous studies of the early time course of labeled mitoses. This is shown in Figure 19. It will be noted that only 55% of myelocyte mitoses were labeled during the first several hours after thymidine injection. Incomplete labeling of mitotic figures may be a consequence of nonuniform availability of precursor, although other factors may also be operative.(1)

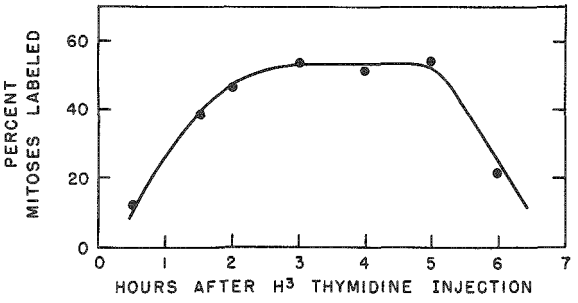

Figure 19

Myelocyte mitoses after single injection of tritiated thymidine.

The duration of the DNA synthetic period has been evaluated in another way in recent experiments. When tritiated thymidine is infused at a constant rate for a period longer than the DNA synthesis time, the degree of incorporation will attain a maximum. Cells in synthesis at the beginning of the infusion will incorporate tritiated thymidine for a variable period. However, a cell that has just entered synthesis will label throughout its synthetic period. Such a cell will label maximally and all cells entering synthesis after the infusion has begun will reach a similar maximum.

Five beagles were infused at a constant rate with tritiated thymidine (H^3T) in 5% glucose. One dog was infused with 12 mc of H^3T (360 mc/mM) over a 12-hr period. The remaining 4 dogs were divided into two pairs: One of each pair received H^3T with a specific activity of 360 mc/mM, the other with a specific activity of 36 mc/mM. All 4 dogs received a total dose of 10 mc of tritiated thymidine, one pair over a 6-hr period, the other over a $6\frac{1}{2}$-hr period. Bone marrow samples were aspirated at frequent intervals during the infusion. Autoradiographs were prepared(2) and one thousand myelocytes in each marrow sample were scored for grain concentration.

Figure 20 shows the myelocyte grain count distribution for one of the dogs. Although grain concentration reaches a maximum with time, several factors must be considered in evaluating data of this sort. Owing to the relatively slow rate of infusion, labeled nucleoside may not be uniformly available at the outset; a few of the heavily labeled cells seen initially may reflect a biologic advantage in opportunity to incorporate the precursor.

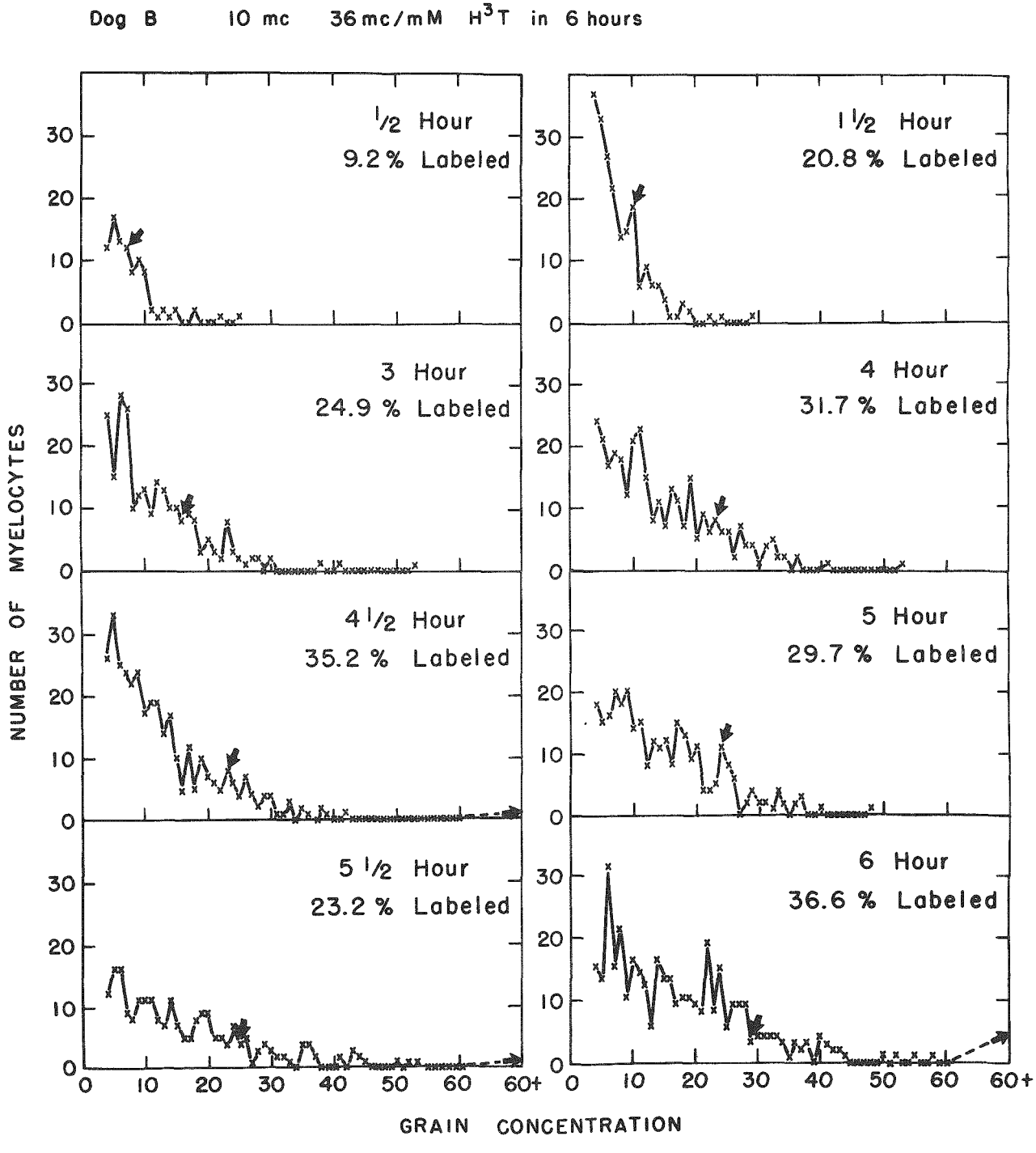

Figure 20. Distribution of myelocyte grain counts during infusion of tritiated thymidine.

More important, perhaps, some of the labeled cells will complete mitosis during the course of thymidine infusion. The progeny of the first of these cells will be minimally labeled because of mitotic dilution and, in consequence, these cells may be lost from the labeled population. However, other cells will be added to the population, as the more heavily labeled myelocytes complete mitosis. Marrow specimens will include cells that are being labeled during the entire period between successive sampling as well as cells that have initiated or completed synthesis in this interval.

In order to circumvent these factors, attention has been focused on representative myelocytes whose relative position in respect to grain count distribution is constant throughout the infusion period; a cell that represents this position is designated by an arrow in Figure 20. The mean grain concentration of such a representative cell in the five dogs which have been studied is shown in Figure 21. Grain concentration increases linearly to a maximum which is reached after $4\frac{1}{2}$ to 5 hr of infusion. Thus it appears that the DNA synthesis period for the myelocytes is $4\frac{1}{2}$ to 5 hr, which is in good agreement with our previous estimate based on analysis of labeled mitoses.

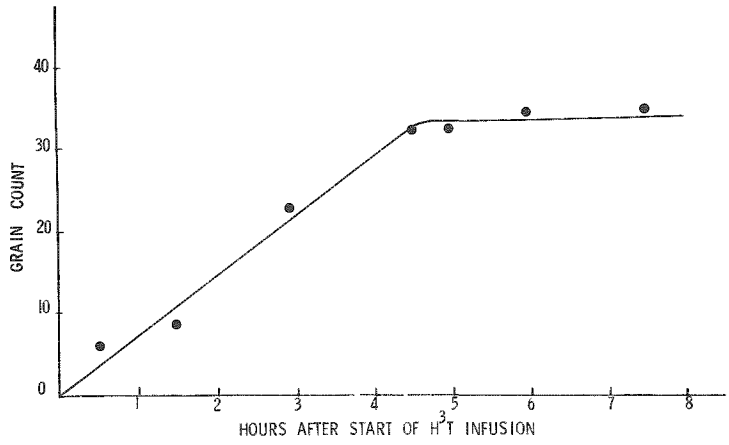

Figure 21

Changes in the grain count of a myelocyte whose relative position is constant throughout the infusion.

References

1. Patt, H. M. and M. A. Maloney. Kinetics of neutrophil balance. In The Kinetics of Cellular Proliferation. New York: Grune and Stratton, Inc., 1959. pp. 201-207.

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

RADIOSENSITIVITY OF THE HYPERTROPHYING MOUSE KIDNEY

R. L. Straube and H. M. Patt

There has been considerable discussion as to the relative importance of hypertrophy and hyperplasia in the renal compensation following unilateral nephrectomy.[1-4] We have employed desoxyribosenucleic acid (DNA) determination in normal and hypertrophying mouse kidney in an attempt to clarify this point. Results indicate that mitosis plays a relatively minor role in kidney enlargement following uninephrectomy in the young adult mouse. This system, therefore, lends itself to study of radiation effects on a growth process which is characterized mainly by enlargement of existing cells.

CF No. 1 female mice of various age groups were anesthetized with Nembutal® administered intraperitoneally. The right kidney was exposed by a dorsal surgical incision, ligated, and removed; care was taken to maintain the integrity of the adrenal gland. Another incision was made, and the left kidney was freed from the adrenal and ovary and exteriorized. Local irradiation of the organ was performed through a 1-cm cone, using a GE Maximar 100 source. The radiation factors were: 100 kv, 5 ma, target distance 15 cm, 2014 r/min using 0.1 mm of Al. By adjusting the X-ray cone and the mouse it was possible to irradiate the exteriorized kidney alone. Precautions were taken to prevent undue pressure upon the kidney or its blood supply during irradiation. The color of the exteriorized organ remained normal throughout the irradiation period. DNA determinations were made according to the method of Schneider[5] as modified by Barton.[6] The unilaterally nephrectomized mice were killed at 5 to 6 days postoperatively and the remaining kidneys were taken for DNA determination. These were compared with the normal kidneys removed from the same animals at the time of operation. The values reported for the 2-month-old animals are based on individual microdeterminations; those of mice in other age groups represent pooled samples of several kidneys.

In the normal mouse, removal of the right kidney results in a 50% increase in wet weight of the left kidney at 26 to 28 days postoperatively. The weight of the remaining kidney at this time corresponds to 75-80% of the initial combined weight of both kidneys. The greater part of this increase (40% increase above initial weight, 70% of initial combined weight) is achieved by 14 days after nephrectomy, and this period was selected empirically as the sacrifice time in the radiation experiments.

In the 2-month-old female CF No. 1 mouse (Table 11) the total DNA content of the normal kidney was 1.12 μg, $\pm$ a standard error of 0.05, whereas that of the kidney that had undergone hypertrophy for 5 or 6 days was 1.26 $\pm$ 0.05. In both the rat[1] and the mouse[2] it has been reported

that mitotic activity, if it occurs in the hypertrophying kidney, takes place within 48 to 72 hr after nephrectomy. Statistical analysis indicates that there was no significant difference between these values and that, in effect, the DNA content of normal and hypertrophying kidney is identical. The presence or absence of mitoses in the kidney of the female CF No. 1 appears to be an age-dependent phenomenon: in pooled samples from 4-, 6-, and 10.5-month-old mice no hyperplasia was seen, but in the young 4- to 6-week group there was some indication of mitotic activity with an increase in DNA content. It may be of interest to note that a similar age-dependent hypertrophy-hyperplasia relationship has been found in the rat.[4]

TABLE 11

DNA content of the residual kidney 5 to 6 days after unilateral nephrectomy

Age at operation	Normal		After nephrectomy	
	No. mice	DNA, μg/kidney	No. mice	DNA, μg/kidney
Pooled samples				
4-6 weeks	13	0.97	13	1.50
2 months	7	1.12	7	1.23
4 months	6	1.04	6	1.07
6 months	3	1.17	3	1.00
10.5 months	11	1.03	11	1.11
Individual determinations				
2 months	9	1.12 $\pm$ 0.05*	9	1.26 $\pm$ 0.04*

*Standard error of mean.

When the contralateral kidney of 2-month-old females was irradiated immediately after uninephrectomy, the hypertrophic response observed at 14 days was not significantly altered until doses of X-ray in excess of 20,000 r were employed (Table 12). The wet weight of kidneys subjected to 30 kr was significantly smaller than that of the hypertrophying kidney without irradiation, but larger than the weight of the normal left kidney. This may be attributed in part to abortive hypertrophy as well as to necrosis and scarring; certainly sufficient functional renal tissue persisted to allow the animals to survive 14 days to sacrifice. When 50 kr was given, renal destruction was sufficient to preclude survival in the majority of mice;

only 1 of 7 animals so treated survived for 14 days. The increased renal damage is probably reflected in the body weight loss evidenced by these animals at the higher dose levels.

TABLE 12

Effect of local kidney X-irradiation after uninephrectomy on renal compensation (measured 14 days postoperatively)

X-ray dose, kr	No. mice	Kidney wet wt., mg (Mean ± S.E.)	p	Body wt. at sacrifice, g	Change in body wt.,* g
Normal kidney	10	132.4 ± 4.0		21.1	
Irradiation immediately after uninephrectomy					
0	18	188.2 ± 4.6		21.4	+0.5
10	10	182.4 ± 4.0	>0.05	20.9	-0.2
20	18	163.6 ± 9.3	<0.05>0.02	17.5	-3.1
30	6	149.6 ± 8.9	<0.001	17.9	-3.0
Irradiation 5 days after uninephrectomy					
0	8	192.4 ± 6.1		21.1	+1.8
10	9	169.7 ± 5.6	>0.05	19.8	-0.6
20	8	161.0 ± 10.4	0.02	18.9	-1.7
30	6	136.9 ± 8.0	<0.001	13.6	-5.8

*Weight at sacrifice minus weight at time of irradiation.

In the compensatory hypertrophy of the kidney following uninephrectomy, the dry renal tissue represents about 25% of the total kidney weight. This ratio remains constant throughout the course of hypertrophy. It is interesting to note that this proportion is not altered in the hypertrophy which accompanies the irradiated nephrectomized state; the dry weight changes follow the wet weight even at the 20- and 30-kr levels.

It was thought that a differential radiosensitivity might exist between the kidney irradiated immediately after nephrectomy and one already undergoing hypertrophy at the time of irradiation. To test this possibility, mice were nephrectomized and then subjected to local kidney irradiation 5 days later. These animals were sacrificed 14 days after irradiation (Table 12).

The picture is similar to that seen when irradiation is administered immediately after nephrectomy; the hypertrophic response shows a significant decrease at about the 20-kr level. Again there is a suggestion of a correlation between extent of renal damage and loss of body weight.

Following our initial observations[3] of the effect of local X-irradiation on the growth capacity of mouse kidney, it was reported[2] that a single sublethal (690 r) dose of X-irradiation to the whole body suppressed mitosis and inhibited subsequent compensatory hypertrophy in LAF_1 mice when given 3 hr after nephrectomy. If radiation was delayed 48 hr until mitosis had occurred, hypertrophy was essentially similar to that of nonirradiated controls. There are obvious differences between local and whole-body irradiation as well as differences between strains, but the discrepancy between these results and ours may also be attributable to age-dependent mechanisms; our 2-month-old CF No. 1 mice had reached a stage of development or differentiation at which hyperplasia no longer played an important part in the kidney enlargement.

It appears, then, that the compensatory response which occurs after unilateral nephrectomy in the young adult mouse is relatively radioresistant and it is tempting to correlate this resistance with the major role that hypertrophy plays in this system. Presumably, the radiosensitivity of cell renewal systems, i.e., organs which, even in the normal steady state, are engaged in continued mitotic activity (marrow and gut epithelium), is, in part, a reflection of greater susceptibility of the mitotically active moiety. In contrast, these studies emphasize the relative insensitivity of a hypertrophying organ to radiation effects.

References

1. Rollason, H. D. Compensatory hypertrophy of the kidney of the young rat with special emphasis on the role of cellular hyperplasia. Anat. Rec. 104 263-285 (1949).

2. Rosen, V. J. and L. J. Cole. Radiosensitivity of mouse kidney undergoing compensatory hypertrophy. Research and Technical Report, USNRDL-TR415 (1960).

3. Straube, R. L. and H. M. Patt. Effect of local X-irradiation on growth capacity of mouse kidney. Radiation Research 11 470 (1959).

4. Kennedy, G. C. Age and Renal Disease in Ciba Foundation Colloquia on Ageing, Vol. 4. London: J. and A. Churchill, 1958. pp. 250-263.

5. Schneider, W. C. Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. J. Biol. Chem. 161 293-303 (1945).

6. Barton, K. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62 315-323 (1956).

PRELIMINARY RESULTS FROM THE MONTE CARLO STUDY IN THE STOCHASTIC THEORY OF MORTALITY

G. A. Sacher and E. Trucco

The stochastic theory of mortality in its present stage of development[1,2,3] deals exclusively with those aspects of the mortality process which are probabilistic in the sense that the occurrence of a lethal event is governed by chance alone.

The oversimplified model studied so far can best be illustrated by a physical analogy: we consider an ensemble of identical and non-interacting particles which are trapped at the bottom of a potential well, i.e., near the point 0 in Figure 22. The particles, however, are also in random (thermal) motion, and this may cause their escape across a potential barrier of height Q (Figure 22). Such an escape is interpreted as the "dying" of a particle.

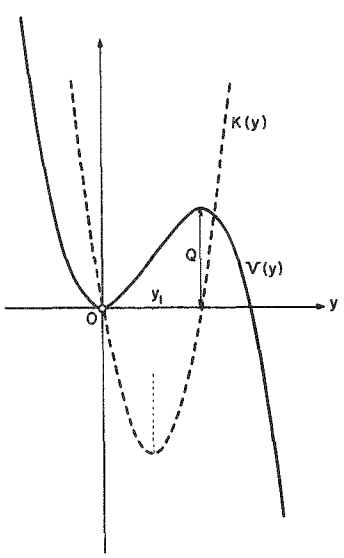

Figure 22

The cubic potential $V(y) = -2Qy^3 + 3Qy^2$ and the restoration function $K(y) = -dV/dy = 6Q(y^2 - y)$.

Clearly, this whole process is a purely stochastic one: we cannot predict with certainty at what time an individual particle will leave the well. But, as Pearson has pointed out,[4] for science chance is identical with knowing the percentage of successes and failures which are sure to occur in a considerable number of trials.

One further point to be emphasized in connection with this simple model is that the potential well may actually be much more than just an analogy. We have, in fact, presented arguments in favor of a physiological potential function to describe the cybernetic behavior of biological organisms (see Section 7 of Reference 3).

The physical problem of particles escaping across a potential barrier was studied by Kramers,[5] who derived equations to calculate the rate of escape in some simple limiting cases. For more general situations, however, an explicit solution cannot be obtained; we have, therefore, found it convenient to use a Monte Carlo computation.

The investigations performed so far are based on a cubic potential function of the form

$$V(y) = -2Qy^3 + 3Qy^2 \quad , \tag{1}$$

where y represents the space coordinate, and Q is a positive constant.

This function is shown in Figure 22 (solid line).

The Monte Carlo program performs a sequence of "trials." Each one of these corresponds to the trajectory of a particle obeying the following equation of motion:

$$\frac{d^2y}{dt^2} = -\beta_2 \frac{dy}{dt} + K(y) + A(t) \quad , \tag{2}$$

where β_2 is a positive constant (the so-called damping coefficient) and A(t) represents a Gaussian random process in time (t) with the properties usually postulated for such functions in the theory of Brownian motion (see Reference 6, Equation 41 and those following). K(y) is the force or restoration function, given by

$$K(y) = -\frac{dV}{dy} \quad ; \tag{3}$$

in our case it becomes a quadratic function of y (Figure 22, dotted line). Beyond the point $y = y_1 = \frac{1}{2}$ the magnitude of the centrally restoring force begins to decrease; it finally becomes equal to zero where the potential function has a maximum.

The initial position and velocity of the particle in each trial are taken at random from a bivariate rectangular distribution of position and velocity centered around the values y = 0 and dy/dt = 0. In most cases an additional initial impulse was also imparted to each particle, as explained below. Thus, in the present approach we have two distinct sources of randomness: one is represented by the function A(t) in Equation 2, and the second is provided by the choice of initial conditions. Even though both of these are undoubtedly present in any natural population, we are, nevertheless, planning to investigate each one of them separately in due course of time.

The first experiments were performed by choosing the initial values as explained above, with the means of initial positions and velocities equal to zero; each trial lasted for the same length of time, t_f. Then the percentage of particles escaping in a group of, say, 100 or 200 trials was counted. In this case it should be possible to compare the results with a theoretical value calculated from an equation given by Kramers[5] (see also Equations 507 and 509 of Reference 7). Kramers' derivation is strictly valid only for the limiting case in which the height, Q, of the potential barrier is very large compared to the variance, Λ_1^2, of the initial distribution of particle velocities.

So far our results have been inconclusive because the observed percentage of escapes was extremely small. To obtain reliable statistics one should therefore take a much larger number of trials and/or a longer time t_f, and this makes the cost of computation prohibitive. We were thus forced to modify the Monte Carlo program, making it more efficient for the case of very low escape probabilities. The procedure which was used is briefly described at the end of this report.

The next series of experiments was performed like the one just mentioned; however, an initial impulse or "kick," of magnitude $c > 0$, was added to the particle's velocity at time zero. This naturally increases the rate of mortality. In a biological interpretation such a kick may represent the effect of a noxious, transient external agent like, for instance, a single dose of ionizing radiation.

As before, the percentage of escapes occurring in a time interval t_f was determined; some first results are shown in Figure 23. Here the magnitude, c, of the kick is plotted along the abscissa, and the percent escapes during t_f is plotted along the vertical axis. Each point is based on at least 100 trials. The resulting distribution of escapes with varying c is approximately a Gaussian error function, as can be seen from Figure 24 where the same data are represented using a probit scale on the ordinate axis.

This result should be compared with the strictly deterministic case which is obtained from the differential equation of motion

$$\frac{d^2y}{dt^2} = -\beta_2 \frac{dy}{dt} + K(y) \quad . \tag{4}$$

Equation 4 was solved numerically on the IBM 650 computer in the Accounting Department; this work was done by Mr. J. A. Scherer whose help is gratefully acknowledged. Choosing the initial conditions $y(0) = 0$ and $(dy/dt)_0 = c$, it is found that according to this deterministic picture <u>all</u> the particles

should stay inside the well if the initial velocity is smaller than a certain threshold c_0 (with the parameter values used in our calculations we find that $c_0 \approx 0.58$, as indicated by the dotted vertical line in Figure 23), and all of them should escape otherwise. Instead of this, however, the number of escapes increases gradually with c as soon as we introduce random terms into our calculations.

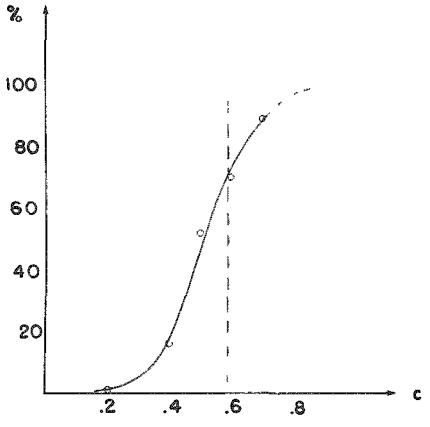

Figure 23. Percentage of particles escaping in a certain time interval t_f plotted versus magnitude, c, of initial impulse.

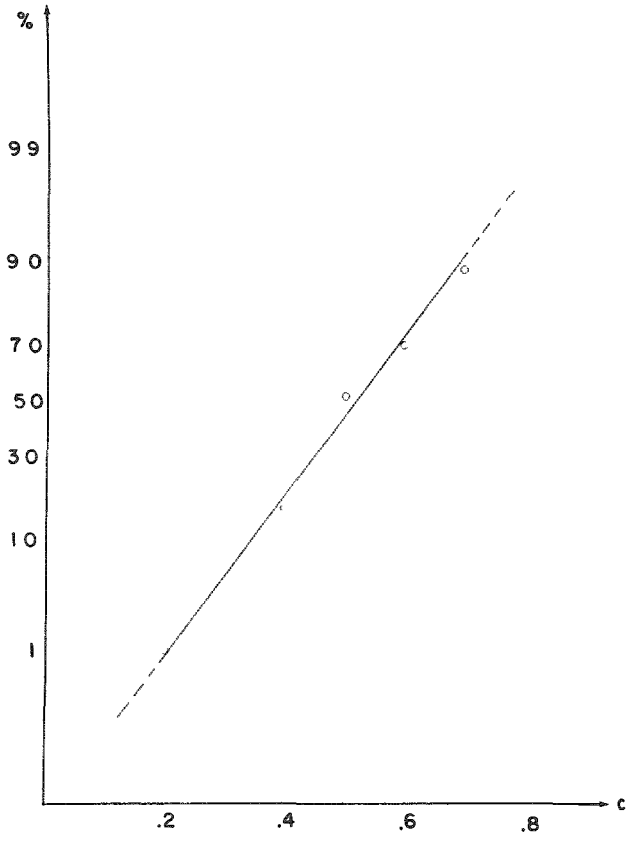

Figure 24. Probit transform of the preceding graph.

When the rate at which particles tend to escape becomes too small, the Monte Carlo program must be improved. This can be achieved by means of a device technically known as "forcing escapes." It is based on the fact that one can change the shape of the potential function within very wide limits, perform the computations with the new potential, and from these results calculate the rate of escapes which would have been obtained with the original potential function. The mathematical details for this, and for other much more general procedures, are given in a paper by Cameron and Martin.(8)

As an analogy we may recall that in some problems it is advantageous to introduce a new system of coordinates for the actual calculations; then, at the end, the results are again expressed in terms of the original coordinates.

Of course, the transformed potential, $\bar{V}(y)$, is chosen in such a way as to increase the probability of escape greatly. We have taken

$$\bar{V}(y) = V(y) - ky \quad , \tag{5}$$

where k is a positive constant, which means that a fictitious centrifugal force of magnitude k is assumed to act on each particle. In this way it will be possible to plot the curves shown in Figures 23 and 24 with a much higher level of precision, especially for small values of c and in particular for c = 0. Some preliminary results obtained by this improved technique are already at hand, but not in sufficient number to be included in this report.

In conclusion we wish to thank Drs. James W. Butler and David A. Woodward of the Applied Mathematics Division who have greatly assisted us in performing this work.

References

1. Sacher, G. A. On the statistical nature of mortality, with especial reference to chronic radiation mortality. Radiology 67 250-257 (1956).

2. Sacher, G. A. Entropic contributions to mortality and aging. In Symposium on Information Theory in Biology, ed. H. P. Yockey. New York: Pergamon Press, 1958. pp. 317-330.

3. Sacher, G. A., and E. Trucco. The stochastic theory of mortality. Ann. N. Y. Acad. Sci., in press.

4. Pearson, K. The scientific aspects of Monte Carlo roulette, In The Chances of Death and Other Studies in Evolution, Volume I. London: Edward Arnold, 1897. pp. 42-62.

5. Kramers, H. A. Brownian motion in a field of force and the diffusion model of chemical reactions. Physica 7 284-304 (1940).

6. Wang, Ming Chen and G. E. Uhlenbeck. On the theory of Brownian motion. II. Rev. Mod. Phys. 17 323-342 (1945).

7. Chandrasekhar, S. Stochastic problems in physics and astronomy. Revs. Mod. Physics 15 1-89 (1943).

8. Cameron, R. H., and W. T. Martin. Transformations of Wiener integrals under translations. Ann. Math. 45 386-396 (1944).

PROGRESS REPORT: ON THE FOKKER-PLANCK EQUATION IN THE STOCHASTIC THEORY OF MORTALITY

E. Trucco

In 1956 Sacher proposed that the following equation, of the Fokker-Planck type, be used to describe random fluctuations of a physiological variable around its mean state:

$$\frac{\partial P}{\partial t} = D \frac{\partial^2 P}{\partial \mu^2} + g \frac{\partial}{\partial \mu} (\mu P) \quad . \tag{1}$$

Here $\mu \equiv x-m$ is the deviation of the variable x from its homeostatically controlled average value m; D and g are constants; $P(\mu,t)d\mu$ represents the probability of observing a certain value μ (within $d\mu$) at time t.

Sacher's basic result was Equation 7 of Reference 1; his approach has been discussed in the literature.[2,3]

However, a closer examination shows that Sacher's calculations should be revised since they are based on a "modified stationary frequency distribution" which actually is not correct. In other words, the quantity $Q(\mu)$ given by Equation 5 of Reference 1 is not a solution of the time-independent equation

$$D \frac{d^2 Q}{d\mu^2} + g \frac{d}{d\mu} (\mu Q) = 0 \quad .$$

A rigorous solution of Equation 1 with appropriate boundary conditions, has, therefore, been obtained by separating the variables t and μ (method of eigenfunctions). It is of the form

$$P(\mu,t) = e^{-\frac{g}{4D}\mu^2} \sum_{n=0}^{\infty} C_n \, v_n(\mu) \, e^{-\lambda_n t} \quad , \tag{2}$$

where the quantities $v_n(\mu)$ are confluent hypergeometric functions (more precisely, functions of the parabolic cylinder), the constants C_n are determined by the initial conditions, and the eigenvalues λ_n can be found graphically.

Let us, in particular, examine the case previously studied by Sacher. We require as a boundary condition that the function $P(\mu,t)$ should vanish for all positive values of t if μ becomes equal to λ:

$$P(\lambda,t) \equiv 0 \quad (t>0) \quad . \tag{3}$$

Here λ is defined as

$$\lambda = L - m \quad , \tag{4}$$

where L is the so-called lethal bound for the original variable x, i.e. an individual is removed from the population if a fluctuation carries its value of x to or beyond L (for details see Reference 1). If we put

$$N(t) \equiv \int_{-\infty}^{\lambda} P(\mu,t)\, d\mu \quad , \tag{5}$$

the rate, ρ, at which members are removed from the population will be:

$$\rho = - \frac{1}{\hat{N}} \frac{dN}{dt} \quad . \tag{6}$$

Still following Sacher we further assume

$$\lambda^2 >> \frac{D}{g} \quad . \tag{7}$$

Then it can be shown that under these conditions:

a) The lowest eigenvalue, λ_0, of Equation 2 will be very close to zero, whereas the next higher eigenvalue, λ_1, is relatively large compared to λ_0. Hence, for values of t which are neither very short nor extremely long we can, to a first approximation, replace the exponential terms $e^{-\lambda_n t}$ by zero if $n>0$, and also put $e^{-\lambda_0 t} \approx 1$. We thus obtain the "quasistatic approximation":

$$P(\mu,t) \approx P(\mu) \approx e^{-\frac{g}{4D}\mu^2} C_0 v_0(\mu) \quad . \tag{8}$$

b) To the same order of approximation it then follows from Equations (6), (5), and (8) that

$$\rho \approx \text{const.}\ \lambda\ e^{-\frac{g}{2D}\lambda^2} \quad . \tag{9}$$

It will be seen that Equation (9) above has the same form as Sacher's Equation 7 of Reference 1. We have, therefore, obtained a more rigorous derivation of this result, so that all the conclusions reached by Sacher in Reference 1 remain unchanged and are now mathematically better justified.

An alternative derivation of Equation (9) consists in following a procedure first used by Chandrasekhar to describe the flow of particles across a very high potential barrier (Reference 4, pp. 63 ff). This again amounts to a "quasistatic approximation" (since the flow in question will be very small).

We shall not, however, give the complete details of our calculations in this report; they will be published shortly in the Bulletin of Mathematical Biophysics.

References

1. Sacher, G. A. On the statistical nature of mortality with especial reference to chronic radiation mortality. Radiology 67 250-257 (1956).

2. Strehler, B. L. Origin and comparison of the effects of time and high-energy radiations on living systems. Quart. Rev. Biol. 34 117-142 (1959).

3. Neary, G. J. Ageing and radiation. Nature 187 10-18 (1960).

4. Chandrasekhar, S. Stochastic problems in physics and astronomy. Revs. Mod. Phys. 15 1-89 (1943).

ON THE KINETICS OF MECHANISMS BY WHICH ACUTE RADIATION INJURY ACCUMULATES

S. A. Tyler and S. P. Stearner

In general, acute effects of external irradiation on animals have been measured by the mortality produced within 30 days of the irradiation. This end point is unsatisfactory as a criterion of injury assessment since by 30 days after an irradiation several mechanisms have been shown to contribute to the resulting mortality. Attempts have been made to distinguish the mortality resulting from each of several injuries present; and many criteria, such as postirradiation time of death, observed pathology and frequency of deaths, have been used to effect a separation. However, only token efforts toward exploring the possibilities of these analytical approaches have resulted because of the severity of criticism that usually follows. These methods do have promise as a means of extracting information from mortality statistics relevant to the kinetics of injury accumulation; and we have been concerned with statistical procedures that are necessary to the accomplishment of this intent.

Essential to effective employment of these procedures is the manner in which the experiment is designed. For example, one motivation behind many acute radiation experiments is the determination of the influence of dose rate on lethality. The experimental design compatible with this aim demands that dose rate be considered as one of the principal, experimentally controlled variables. From radiation experiments with young chicks, chick embryos and mice under single, protracted exposures, it has been found that a reduction in radiation effects takes place during an exposure, and the amount of the reduction can be exhibited as a simple function of the time over which the dose is protracted. Thus, exposure time seems to be more important as a descriptive variable than dose rate in this instance. The use of dose rate may add to the complexity of an empirical description of acute radiation kinetics since the rate is a composite of two variables - total dose and exposure time.

Selection of the quantity (or quantities) by which reduction in dose effectiveness is to be measured is extremely important. It is seldom that a "best" measure can be chosen; however, a preliminary scrutiny of suggested measures of effect can insure that a mathematical artifact is not introduced into the descriptive analysis. For example, reduction in dose effectiveness with time has been measured as the ratio of dose remaining ($D_{j,m}$) after some interval of time (t) and dose delivered ($D_{k,m}$) for some criterion of effect (m).

$$\frac{D_{j,m}}{D_{k,m}} = f(t) \quad , \tag{1}$$

where f(t) is some function of t. This ratio is frequently used with split-dose exposures to indicate the proportion of the initial dose that remains at the time a second exposure is administered. Only when the initial dose is held constant does this expression provide a meaningful description of the relationship between dose reduction and time. This requirement is not always observed. In the case of protracted exposures, $D_{j,m}$ represents the instantaneous dose (abstractly, a dose delivered in an infinitesimal interval of time) required for a criterion effect and $D_{k,m}$ is the total dose required for the same effect when protracted over time t. In this case, $D_{k,m}$ must of necessity vary with t. By use of the ratio in Equation 1, however, demonstration of a linear reduction in dose is precluded. To illustrate, let us assume that a dose reduction process is, in fact, described by the linear expression

$$D_{j,m} = D_{k,m} - \alpha t \quad , \tag{2}$$

where α is the constant rate of reduction and $D_{j,m}$ is the dose equivalent to the insult producing effect m. Then

$$\frac{D_{j,m}}{D_{k,m}} = \frac{D_{j,m}}{D_{j,m} + \alpha t} = \frac{1}{1 + St,} \tag{3}$$

where $\alpha = SD_{j,m}$. Thus dose reduction as defined by Equation 3 is nonlinear. This violates the assumption of linearity that is expressed by Equation 2. In a demonstration of the dependence of dose reduction on exposure time for a single protracted exposure, the reciprocal ratio $D_{k,m}/D_{j,m}$ will not obscure the kinetics of processes under consideration, regardless of their mathematical form, since the denominator of this ratio is the same for all values of t.

Even after obvious pitfalls have been avoided in the choice of the experimental design and the variables upon which the statistical description will depend, several working models of effects that are equally compatible with experimental data can usually be inferred by appraising the quantitative analysis. Let us examine this point by considering only the acute mortality within 3 to 6 days after irradiation (second period) among white leghorn cockerels that were exposed to γ-rays on the third or fourth day after hatching (Figure 25).* The percent mortality within this period, based on the number alive on the third day, is a measure of the lethal actions of some injury process that is assumed to contribute independently to the total, acute radiation insult.

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*See references 1-3 for results in the 0 to 2 day (first) period.

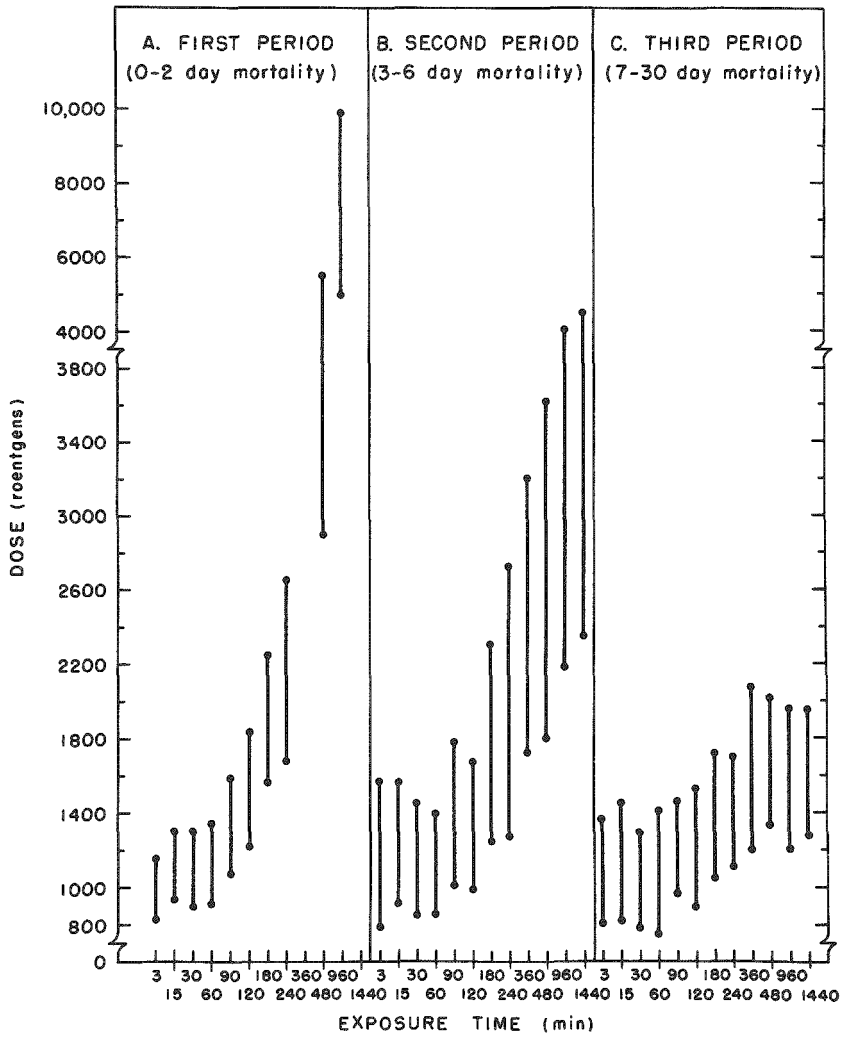

Figure 25. The 10-90% range of radiation mortality for 3 periods that span the first 30 days after irradiation.

After an initial lag for exposures of 60 min or less (Figure 26), the effect of protraction upon the dose distribution of mortality is unmistakable. For exposures of 960 min or longer, the exposure-time effect has practically disappeared and the stationary dose-mortality relationship is approximately given by the 960-min group. Of the many possible interpretations of this empirical behavior, we have elected to investigate first the consequences of the following assumption:

> The mortality due to radiation in the second period is the lethal consequence of the combined but independent actions of two distinctly different injury mechanisms: a reversible process that is dominant for short exposures and an irreversible process that is principally responsible for the observed lethality after long exposures.

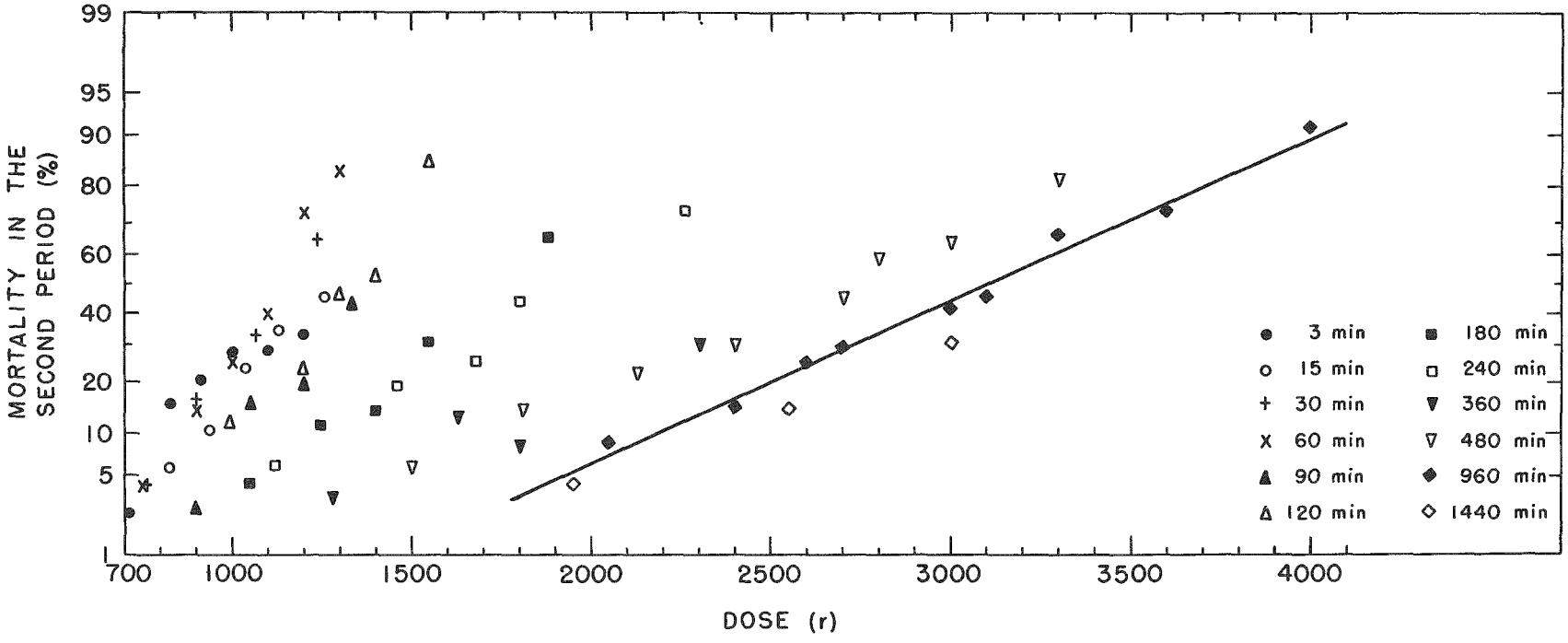

Figure 26. Percent mortality within the second period of the chicks entering this period alive versus dose - by exposure-time groups. The least square line of the 960-min group is given.

Our aim is now to separate these two effects and describe their individual contributions to the observed mortality. Let $R_{d,t}$ represent the expected total probability of death in this period from a dose d that was uniformly delivered over t units of time, and $P_{d,t}$ and Q_d the proportions expected to die from reversible and irreversible effects, respectively. Then,

$$R_{d,t} = P_{d,t} + Q_d - (P_{d,t})(Q_d)$$

$$= P_{d,t} + Q_d(1 - P_{d,t}) \quad , \tag{4}$$

and

$$P_{d,t} = \frac{R_{d,t} - Q_d}{1 - Q_d} \quad . \tag{5}$$

The observed proportion dying in this period is an estimate of $R_{d,t}$, and the mortality proportion for a dose d from the least squares probit line of the 960-min group (LD_{50} = 3105 r, $\hat{\sigma}$ = 717 r - Figure 26) is substituted for Q_d. Using Equation 5, the effects due solely to reversible injury processes can be exhibited (Figure 27).

The distribution of sensitivities to reversible effects is proportional to the distribution of mortalities when the dose is given over so short an interval of time that the reduction in dose-effectiveness is negligible. This distribution was approximated by least squares using mortality data from the 3-, 15-, 30- and 60-min exposure-time groups combined (LD_{50} = 1185 r, $\hat{\sigma}$ = 273 r - Figure 27). Let D(t) represent the dose given over an exposure time t that produces some level of mortality from reversible effects alone and D_0' equal the dose required for the same mortality if given in an infinitesimal interval of time. The quantity $D(t)/D_0'$ was computed for each irradiated group and the mean ratio for each exposure time was calculated and plotted (Figure 28). It is seen that a linear relation approximately describes the dependence of the mean ratios on exposure times that exceed 30 min. Thus, after an initial lag in reversal of from 30 to 60 min, a linear dose-reduction process characterizes injury accumulation by time-dependent processes of this period. The point estimate of the constant τ' is 290 ± 16 min. This value is quite close to the corresponding estimate of 265 ± 33 min calculated for the irradiated chick embryo.(4)

Other models of injury accumulation that comprise the acute radiation syndrome are being considered, and a comparative study of the acute mortality patterns in the chick, parakeet and pigeon is under way.

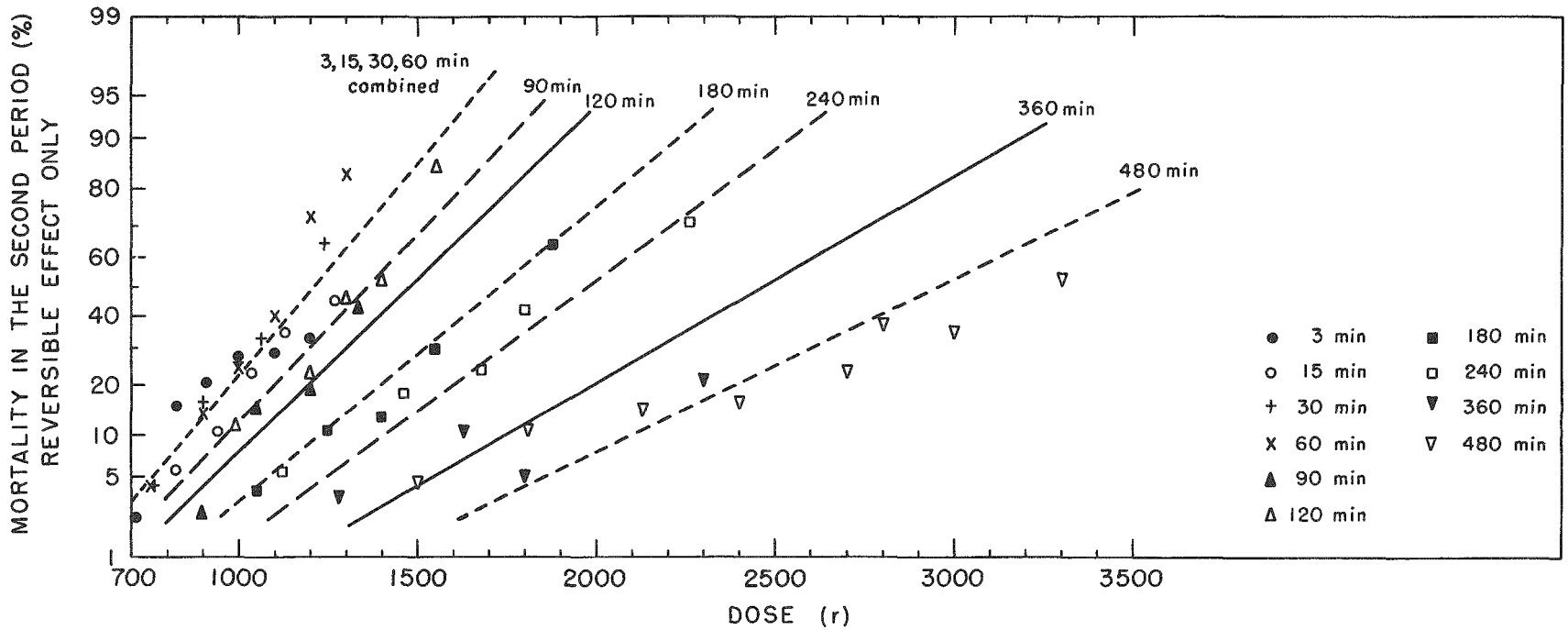

Figure 27. Dose-mortality curves by exposure-time group for reversible effect only. Theoretical lines for each time group are given.

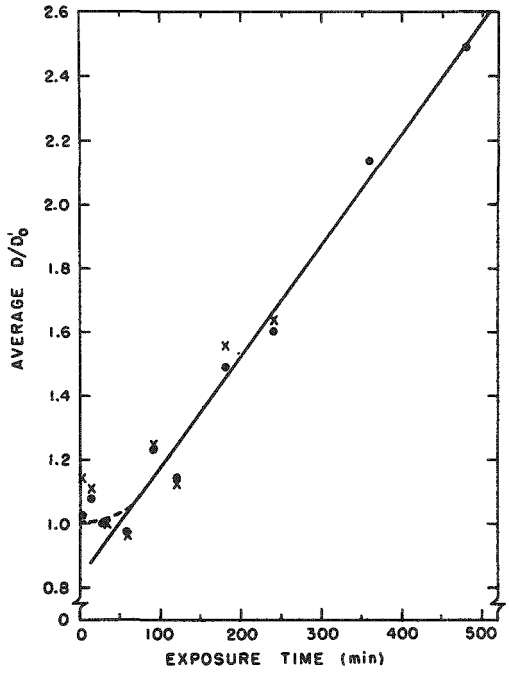

Figure 28

Average D/D_0' versus exposure time. The solid circles represent D/D_0' averages for the second period.

References

1. Stearner, S. P., and S. A. Tyler. An analysis of the dependence of the radiation response in the chick on dose and exposure time. Quarterly Report of Biological and Medical Research Division, Argonne National Laboratory. ANL-5576 (1956), pp. 61-63

2. Tyler, S. A., and S. P. Stearner. A tentative model of the 0-2 day acute radiation response in the chick. Biological and Medical Research Division Semiannual Report, Argonne National Laboratory. ANL-5732 (1957), pp. 75-80

3. Stearner, S. P., and S. A. Tyler. An analysis of the role of dose and dosage rate in the early radiation mortality of the chick. Radiation Research 7 253-266 (1957)

4. Tyler, S. A., and S. P. Stearner. Modes of radiation death in the chick embryo. II. A model of lethal mechanisms. Radiation Research 12 301-316 (1960).

THE UPTAKE AND RETENTION OF DIETARY STRONTIUM-90

M. P. Finkel, P. J. Bergstrand, and D. J. Graubard

Since Sr^{90} has become an integral part of our diet, its incorporation into the skeleton is assured. The experiment reported here was concerned with the rate at which this radioelement accumulated in the body of mice when it was a constant constituent of the diet and with the rate at which it was eliminated when standard food was once again supplied. The variable under investigation was age.

Materials and Methods

The experimental design is given in Table 13. The first group of mice (strain CBA) were conceived after their dams had been placed on food containing Sr^{90}, so their initial age is listed as -20 days. Their strontium was derived first through the placenta and then through suckling. At about 15 days of age, when their eyes are open and they can move about the cage quite readily, mice begin to eat solid food. However, some will continue suckling up to 5 weeks of age if the opportunity is present. In this experiment the dams were separated from their litters on the 28th day of age. The second group of mice, along with their dams, were given food containing Sr^{90} when they were 14 days old. Consequently, their source of strontium between 14 and 28 days was both from solid food and from milk. The other groups received all their strontium from the solid food since they were started on the diet at 30, 70, and 150 days of age.

TABLE 13

Experimental design

Group	Initial age (days)	Total number of animals	Food $\left\{\frac{\mu c\ Sr^{90}}{gm\ Ca}\right\}$	Return to standard diet		
				Number of mice	Age (days)	Days on diet
1	-20	54	5.0	15	28	48
2	14	30	5.0	7	49	35
3	30	37	10.0	8	63	33
4	70	27	10.0	7	103	33
5	150	24	10.0	10	183	33

Two dietary levels were used. Since the Sr^{90} content of living mice was to be determined by measuring brehmsstrahlung with a NaI crystal, higher counts were advantageous. However, it was feared that

metabolic disturbances might occur in the rapidly growing mice at levels that would be optimum for counting. As a compromise, the first two groups of mice received food containing 5 μc Sr^{90}/g Ca, while the others were maintained on diets with twice as much radioactivity. To permit direct comparisons among groups, all Sr^{90} values for groups 1 and 2 have been doubled. During the course of the experiment, the Sr^{90} content of 70 mice was determined by ash analysis so that the brehmsstrahlung measurements could be converted to microcuries.

The food was prepared by adding Sr^{90} (in equilibrium with Y^{90}) to standard, commercially available mouse food before it had been pelleted. Pellets of the usual hardness were then produced by a laboratory-size pelleting machine. The calcium content of the ground meal was 1.76% so each gram of finished food contained 0.176 or 0.088 μc Sr .

A few mice from each group were returned to the standard diet after they had been eating Sr^{90} for more than a month. The remaining animals continued on the special diet.

Results

The most rapid accumulation of Sr^{90} occurred in the mice that were 14 days old initially (Figure 29). During the first 28 days the increase was linear at the rate of 0.032 μc/day.

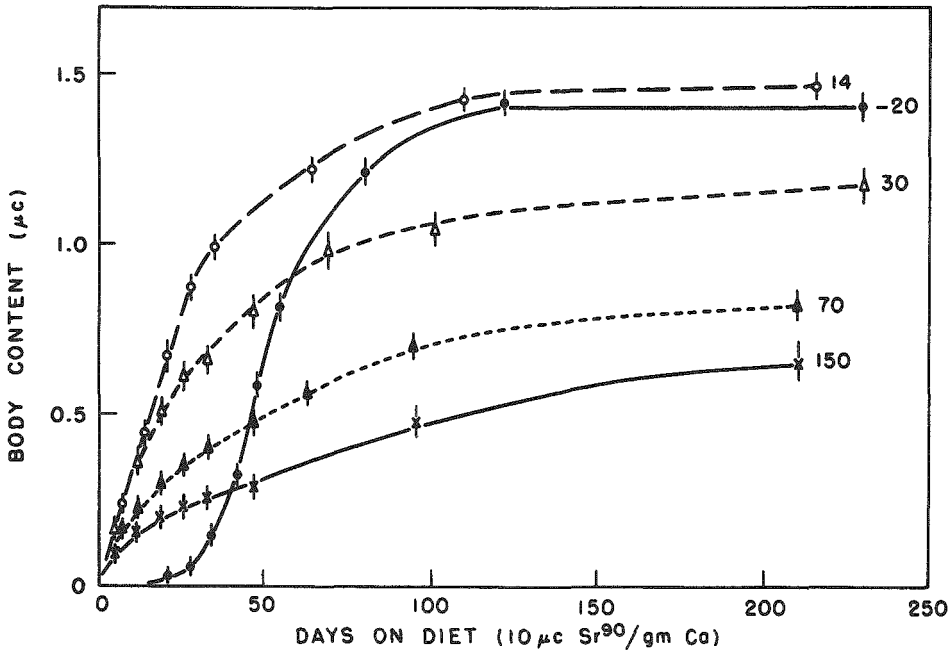

Figure 29. Body content of Sr^{90} as a function of time. Numbers refer to age when ingestion of the radioactive food began.

*California Pellet Mill Co., San Francisco, California.

Accumulation of Sr^{90} within this group of animals became progressively less as they grew older, and it was negligible after 110 days. Among the older animals, the rate of accumulation decreased as their initial age increased, but the uptake of Sr^{90} was still significant long after 100 days. The mice that were exposed to Sr^{90} from conception contained only a small amount at birth, and they gained relatively little during the first week after birth. However, after the first week accumulation was rapid, and at about 100 days their values approached those of the group that had been 14 days old when the strontium diet was first provided. There was no increase in the body content of the animals started at -20 days between 122 and 230 days of age.

The same data are plotted in Figure 30 on a log-log grid with age rather than days on the diet as the abscissa. This representation points out the fundamental similarities among the 5 curves, and it places the -20 day group in its proper place. It seems that these animals, and the 14-day group as well, had reached equilibrium at least by 200 days. However, there is little likelihood that mice started on a radiostrontium diet at 30 days of age would reach equilibrium during a normal mouse life span. A straight extension of the data reaches 1.4 μc, the equilibrium level of the first group of mice, at about three years of age.

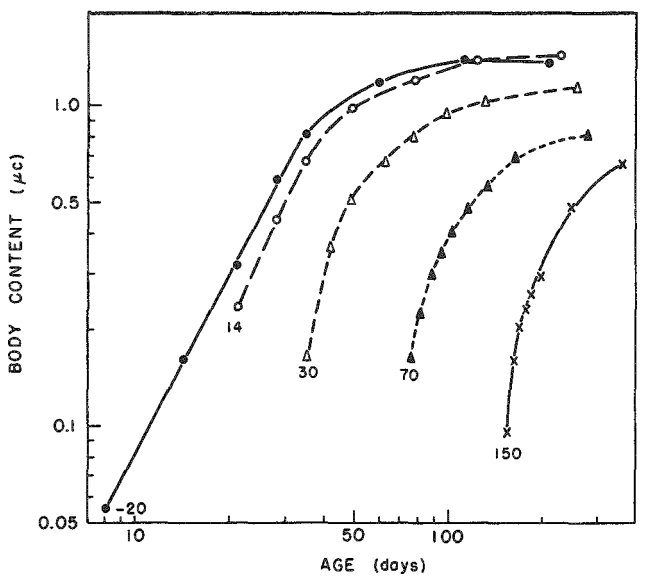

Figure 30

Body content of Sr^{90} as a function of age. Numbers refer to age when ingestion of the radioactive food began.

When the logarithm of body content is plotted against the logarithm of days on the diet (Figure 31), some reasonably straight lines result. The slopes of the curves were much more steep when the animals were young, and, except for the mice that were 150 days old initially, the slopes changed as each group of animals aged. Some of the mice have remained on the experimental diet, so it should be possible at some later date to extend these curves beyond a year.

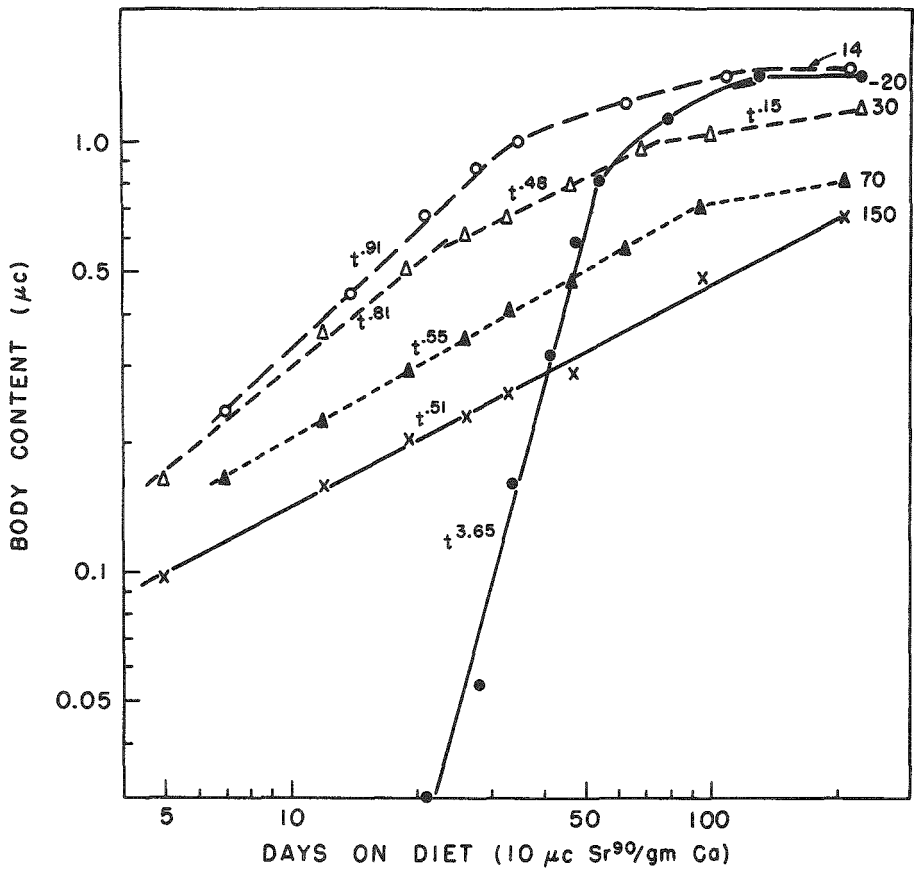

Figure 31. Rate of accumulation of Sr^{90} as a function of time. Numbers refer to age when ingestion of the radioactive food began.

At four weeks of age some of the mice that had been exposed to Sr^{90} since conception, or for a total of 48 days, were placed on the standard food. Thirty-two days later their Sr^{90} content had been reduced by 27%. The curve as drawn during this interval in Figure 32 is purely speculative; very likely the largest part of the decrease occurred during the first 24 hours, while the contents of the digestive tract were being changed from food containing Sr^{90} to standard food. Some mice of the other groups were returned to standard food after they had been eating Sr^{90} for 33 or 35 days. In general, their "wash-out" curves are similar, but there is one striking exception. Retention seemed to be much greater among those animals that were removed from the diet at 63 days of age. This fact is emphasized when the values are compared on a percentage basis, as has been done in Figure 33. The remarkably high retention among the 63-day-old mice agrees with the data of Speckman and Norris on the retention of injected Sr^{85} in the rat as a function of age.(1) Except for this group, retention decreased with increasing age, as expected.

Although retention during the first 2 weeks was greatest among this third group of mice, their subsequent excretion rate was not too different from the mice that were removed from the strontium diet at 103 and 183 days of age.

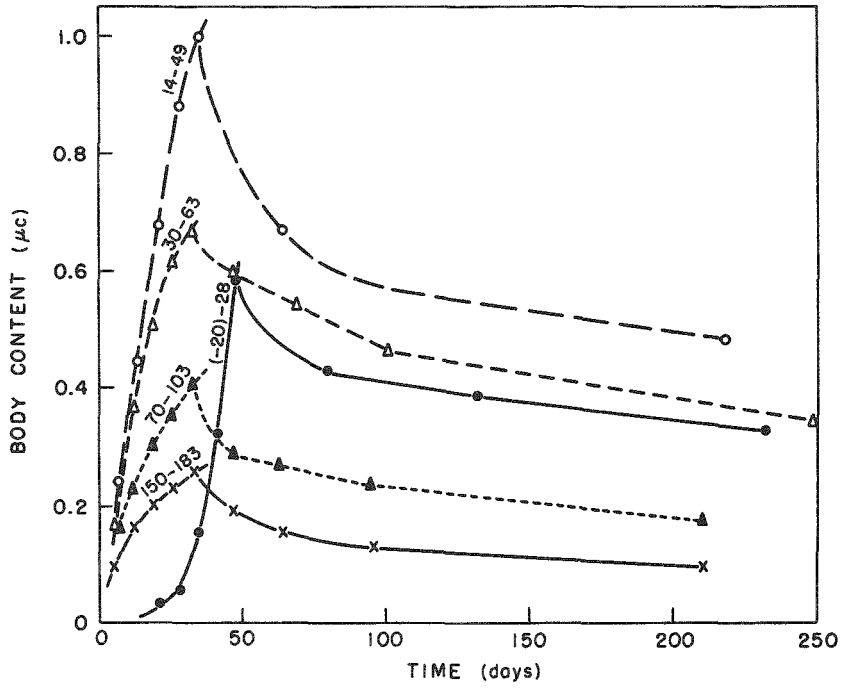

Figure 32. Accumulation and retention of dietary Sr^{90}. Numbers refer to the ages during which the radioactive food was eaten.

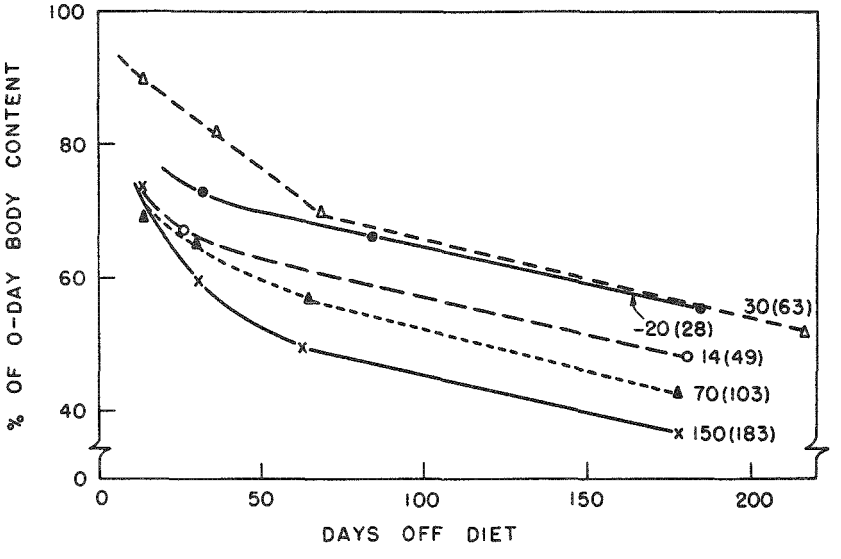

Figure 33. Retention of dietary Sr^{90} as a function of time elapsed since it was ingested. Numbers refer to ages during which the radioactive food was eaten.

As plotted in Figure 34, only the data of the oldest group can be described adequately by a single power function. Functions for the other groups were calculated in order to provide a rough measure of the slopes for comparative purposes. The sequence is more or less as expected. That is, the excretion rate was higher among the animals that were older at the time they returned to the standard diet.

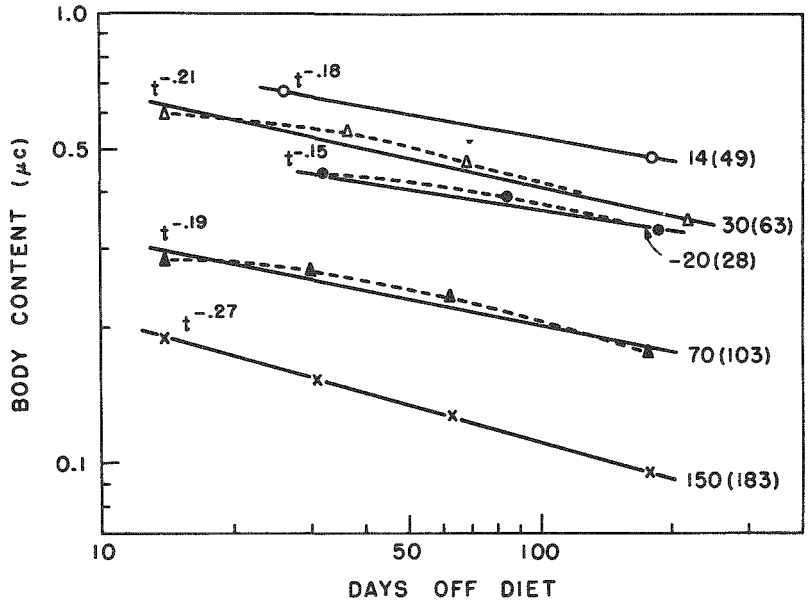

Figure 34. Rate of excretion of Sr^{90} as a function of time elapsed since it was ingested. Numbers refer to ages during which the radioactive food was eaten.

It is interesting to compare these excretion rates after ingestion over a 33- or 35-day interval with those following a single injection. Speckman's data after a single injection of 28-day-old mice with Sr^{85} follow a slope of $t^{-0.17}$.[2] His value for 392-day-old mice is $t^{0.32}$. These are not too different from the estimates obtained here. However, our own data on injected 70-day-old mice, as calculated by Tyler,[3] follow a slope of $t^{-0.32}$. This rate of elimination is much more rapid than that observed in the present experiment for animals of comparable age, i.e., $t^{-0.21}$ for mice maintained on strontium food between 30 and 63 days of age, and $t^{-0.19}$ for those on the diet between 70 and 103 days of age. These differences associated with conditions of exposure, that is, single injection versus continuous ingestion, deserve further investigation since we are faced with the necessity of estimating the human hazard from contaminated food.

Summary

CBA mice of five age groups were provided with food containing radiostrontium, and their Sr^{90} content was determined by brehmsstrahlung counting. Animals started on the diet either at the time of conception or when they were 14 days old showed the greatest accumulation of Sr^{90}. However, their body burden increased very little, if at all, after 100 days. The rate of uptake decreased with increasing age at the time the special diet was first provided and also with increasing age as the animals remained on the diet. Mice placed on the diet at 30 days of age or older contained significantly more Sr^{90} at 200 days than they had after being on the diet for 100 days, and it appeared that the normal life span of a mouse was not long enough to permit equilibrium to be attained.

The retention of radiostrontium that had been supplied in the diet for over a month was followed in some animals that were returned to the standard food. Except for the exceedingly high retention among the mice that were 63 days old when normal food was provided, retention 2 weeks later was inversely related to age. Similarly, the excretion rates seemed to increase as the mice aged, except for the oldest animals, which were on the strontium diet between 150 and 183 days of age.

The excretion rate for mice on a strontium diet from 30 to 63 days of age was calculated to be about $t^{-0.21}$. That for mice on the diet from 70 to 103 days of age was found to be about $t^{-0.19}$. These rates are much slower than the $t^{-0.32}$ observed for 70-day-old mice after a single intravenous injection of Sr^{90}.

References

1. Speckman, T. W. and W. P. Norris. Strontium-85 retention by the rat as a function of age at injection. Biological and Medical Research Division Semiannual Report, Argonne National Laboratory. ANL-6093 (1958), pp. 82-87.

2. Speckman, T. W. Personal communication.

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

THE $LD_{100/35}$ FOR Sr^{90} IN THE EVERGLADES RAT SNAKE (ELAPHE OBSOLETA ROSSALENI)

L. S. Lombard, C. F. Decker and A. M. Brues

In the course of determining whether Sr^{90} induces neoplastic changes in the skeleton of the snake, it was necessary to ascertain the lethality of a given dose for the species of snake to be used. In this connection it was of interest to compare acutely toxic levels for the snake with those for other species, for example, the mouse, which has a $LD_{50/30}$ for Sr^{90} of 6 mc/kg,[1] the rat, 2.5 mc/kg,[2] and the dog, 150 μc/kg.[3] The present report also includes observations on the clinical and gross pathologic alterations occurring with one given dose. Measurements of radioactivity in tissues and excreta, as well as microscopic alterations in the tissues, will be reported in the future.

Materials and Methods

Three male and 3 female adult Everglades rat snakes (Elaphe obsoleta rossaleni),* while anesthetized, were injected intracardially through a laparotomy incision with 1 μc of Sr^{90}/g body weight.

The 6 snakes and one control were not fed for at least one week prior to injection. Anesthesia was induced by injecting Nembutal®, Abbott (pentobarbital sodium, 1 grain/cc) into the left lateral aspect of the pleuroperitoneal cavity at the upper midportion in order to avoid the lung sac and heart; the dose was 0.03 mg/g body weight.[4] Anesthesia was considered complete when the righting reflex was lost, and the time required for this varied from 5 to 30 min. Although the recovery period was not established, the snakes had not revived 5 hr later but were fully recovered when observed 20 hr afterwards.

The heart was exposed in the anesthetized animal by a right ventro-lateral incision which began approximately 15 to 20 cm from the base of the skull and extended 10 to 15 cm caudad. The location of the heart varied with the length of the specimen, the base being 26.5 cm from the base of the skull in the longest specimen and 20 cm in the shortest one. Blood smears were prepared at the time of surgery from all but one animal and were examined later for parasites.** Sr^{90} in equilibrium with Y^{90} (2.47 mc/ml in isotonic saline at a pH of 5 to 6) was injected with a 27-gauge needle into the ventricle of the heart. Table 14 lists the doses.

*Identification of the species of rat snake was made by Robert Inger, Chicago Natural History Museum.

**Examinations for blood parasites were made by Marietta Miller, Argonne National Laboratory.

TABLE 14

Weight changes in snakes injected with Sr^{90}

Snake no.	Sex	Nembutal, mg	Sr^{90} (2.47 μc/ml), ml	Survival, days	Body weight at time of injection g	Weight change,* g	Combined skeletal and muscle weight at death, g
1	M	21.5	0.35	31	711	-85	401
2	F	26.0	0.45	24	862	-321	294
3	M	20.0	0.33	35	668	-50	378
4	F	20.0	0.33	27	681	-111	335
5	M	20.0	0.33	19	757	-112	358
6	F	20.0	0.33	35	662	-251	242
7	M	18.5	-	55**	615	+117	401

*Weight at time of death minus weight at time of injection.

**Killed at 55 days after injection of saline solution.

Following injection the incision was closed with number 3 nylon suture thread (Vetafil, Bengen), by using a continuous-suture stitch. The snakes were placed in separate plastic rat cages previously lined with heavy plastic sheeting held in place by ignition tape. The plastic liners together with excreta were collected for ashing.

The control animal was operated on in a similar manner, twice within 3 days, and injected each time with 1 ml of physiologic saline (0.85%). It remained alive and in excellent condition for the duration of the experiment (Table 14, No. 7).

Necropsies were performed on all snakes. Organ weights were obtained on the skin, skeleton with attached musculature, heart, liver, gall bladder, spleen, kidneys and gonads. After tissues were taken for histologic sectioning, the remaining organ tissues were weighed and ashed for future studies. Bacterial cultures of the livers of 3 snakes (Nos. 1, 2, 3) were prepared in tetrathionate broth and plated on Salmonella-Shigella media (SS).

The skeleton with attached musculature of an additional adult male specimen was digested free of muscle by beetles of the family Dermestidae at the Chicago Museum of Natural History. The skeleton thus cleaned of all apparent muscle and cartilage was found to weigh one-thirteenth of the total body weight.

Results

Within 2 to 3 days following injection with Sr^{90}, two females (Nos. 2 and 6) laid eggs which were measurably radioactive. All 6 reptiles failed to eat after the second week following injection. The 2 snakes dying on the 35th day developed subcutaneous edema of the head and upper one-third of the body 3 days prior to death. Blood-tinged fluid dripped from the mouth, which could not be closed owing to a marked swelling of the jaws. Three snakes died within 30 days and all 6 died within 35. The specimen that died on the 19th day (No. 5) had the most severe infection with a protozoan, a Haemogregarina species, which is found in the erythrocyte. Two snakes (Nos. 3 and 6) had a light infection with the parasite, while two others (Nos 1 and 2) had none. The blood of snake No. 4 was not examined.

The radioactive eggs began to shrivel and change from white to orange by the third day after being laid, whereas normal eggs from the same species of snake showed no color change or shriveling until some 5 weeks later.

At necropsy hemorrhages varying from petechial to diffuse were found in the subcutaneous and muscular tissue of all animals, being particularly concentrated in the head and tail. Hemorrhages were also observed in the visceral organs, particularly in the lungs, heart, liver, stomach, intestines and kidneys. In the females, there were hemorrhages in the walls of the oviducts. The spleens were small, weighing approximately one-fourth as much as those of the controls. A gray-white concentric ring could be found at the injection site in the 3 snakes dying from the 19th to the 27th day, but no lesions could be distinguished at the approximate injection site in those dying from the 31st to the 35th. Extensive hemorrhages in the musculature in close proximity to the ribs and vertebrae were noted in animals dying from 24 to 35 days after injection.

Multiple gray-white foci were seen in the kidneys of one snake (No. 4) and similar lesions appeared in the lungs of another (No. 1). The livers of 3 snakes (Nos. 3, 4, 6) had from one to three gray-white areas. Bacteriologic cultures of the livers of the first 3 snakes were negative.

Summary

Six snakes were injected intracardially with Sr^{90}, 1 μc/g body weight. Clinical signs were anorexia, weight loss, subcutaneous edema and bleeding from the mouth. All died within 35 days. At necropsy, the spleens were small and hemorrhages occurred in the visceral organs, subcutaneous and intramuscular tissues.

References

1. Finkel, M. P., and G. M. Scribner. Toxicity of strontium-90 and of calcium-45 in mice. I. Status of experiments 200-300 days after injection. Report on Biological, Medical and Biophysics Programs, Argonne National Laboratory. ANL-5456 (1955) pp. 36-41.

2. Anderson, W. A. D., G. E. Zander, and J. F. Kuzma. The study of toxic doses of strontium-90 in the adult rat. A.M.A. Arch. Pathol. 62 433-440 (1956).

3. Finkel, M. P., and A. M. Brues. Sequelae of radiostrontium administration to dogs. Radiation Research 3 224 (1955).

4. Karlstrom, E. L., and S. F. Cook, Jr. Notes on snake anesthesia. Herpetological Notes No. 1. 57-58 (1955).

CYTOCHROME C OXIDASE ACTIVITY IN AMOEBAE

J. F. Thomson and E. W. Daniels

Homogenates of Pelomyxa carolinensis, Pelomyxa illinoisensis, and Amoeba proteus were assayed for cytochrome c oxidase to see whether there might be any correlation between the levels of this enzyme and the radiosensitivity of these amoebae.

The results indicated that both species of Pelomyxa contained approximately the same amount of activity although their radiosensitivities differ by a factor of 10. A. proteus, the most radioresistant of the three species examined, had only one-tenth as much cytochrome oxidase activity as the two giant amoebae.

This enzyme is extremely labile, its activity falling to about 10% of the original level in 40 min at room temperature. This lability is more marked in P. carolinensis than in P. illinoisensis. Since cytochrome c has not been found in amoebae, it is doubtful whether the enzyme was acting on a physiological substrate. Nevertheless it is clear that these organisms contain an enzyme capable of catalyzing the oxidation of molecular oxygen of reduced cytochrome c from mammalian sources.

PRELIMINARY REPORT: SPECTROPHOTOMETRIC TITRATION OF DEOXYRIBONUCLEIC ACID

L. G. Bunville

In aqueous solutions of sufficiently low pH, deoxyribonucleic acid (DNA) undergoes a transition from the ordered, double helical configuration of the native molecule to the disordered configuration characteristic of the denatured molecule. This transition, occurring in a very narrow range of pH, is the result of electrostatic repulsions between protons bound to the base residues of cytidine, adenosine and guanosine, and is accompanied by a large increase in ultraviolet absorption and an increase in the number of protons bound. The pH at which the transition occurs is dependent upon the composition of the DNA.(1)

As a means of elucidating the transition in acid solution, potentiometric and differential spectrophotometric titrations have been carried out for DNA samples of varying composition. The potentiometric titration curves cannot be readily interpreted owing to uncertainties regarding the parameter describing the electrostatic interactions between DNA-bound charges and free protons. This difficulty can be overcome through combination of the differential spectrophotometric and potentiometric titration curves to obtain the change in ultraviolet extinction coefficient as a function of the number of protons bound. Analysis of the data is not complete, but the linearity of the curves so obtained suggests that the dissociation constants for adenine and cytosine in native DNA are very similar.

The slopes of the curves also give the change in extinction upon binding one mole of hydrogen ions to these nucleoside residues. This quantity, change in extinction per mole of bound protons, has been obtained for the various samples of DNA, and allows an estimate of the differential extinction coefficients of the individual bases to be made by extrapolation to the limits of composition, corresponding to adenine-thymine, or guanine-cytosine DNA. The differential extinction coefficients so obtained appear to be about 60% of the value for the free nucleosides, possibly indicating a greater hypochromism in the protonated base residue than in the neutral residue in DNA.

Complete analysis of the data should lead to a description of the distribution of protons in native DNA just prior to denaturation.

Reference

1. L. G. Bunville and E. P. Geiduschek. Biochem. Biophys. Res. Comm. 2 287 (1960).

PREPARATION AND PROPERTIES OF S-ADENOSYL-L-HOMOCYSTEINE, S-ADENOSYL-L-HOMOCYSTEINE SULFOXIDE, AND S-RIBOSYL-L-HOMOCYSTEINE

John Duerre

S-Adenosyl-L-homocysteine was first characterized by Cantoni and Scarano[1] as a demethylation product of S-adenosyl-L-methionine, and the structure they suggested was verified by total synthesis by Baddiley and Jamieson;[2] de la Haba and Cantoni[3] described the enzymatic synthesis of this compound from adenosine and homocysteine by a condensing enzyme prepared from rat liver:

Adenosine + L-homocysteine $\rightleftharpoons$ S-adenosyl-L-homocysteine + H_2O

However, as yet, there are few reports of the biological function or fate of this compound. This paucity of data has been due partly to the difficulty of obtaining sufficient amounts of S-adenosyl-L-homocysteine at a reasonable expenditure of time and starting material.

This paper reports a simplification of the procedure of de la Haba and Cantoni[3] for the preparation of S-adenosyl-L-homocysteine and a study of the chemical properties of the compound. Two derivatives of S-adenosyl-L-homocysteine were prepared and characterized. It is believed that the present observations will facilitate further biochemical study of these compounds.

Experimental

Enzyme. Rat liver homogenates were prepared following the description of de la Haba and Cantoni.[3] The crude homogenates were stored at -10°C until used.

Compounds. Adenosine used in the preparation of S-adenosyl-L-homocysteine was obtained from California Biochemical Research Foundation and DL-homocysteine (free base) was obtained from Nutritional Biochemical Corporation. An authentic sample of S-adenosyl-L-homocysteine for use as a reference compound was obtained from Dr. Leo Parks, Oregon State College, Corvallis, Oregon. $BaCO_3$ was prepared by dissolving 7.5 g $Ba(OH)_2$ in 100 ml of boiling water, cooling and then adding dry ice until neutral. After the precipitate had settled, most of the supernatant fluid was removed by decantation.

Preparation of Ion Exchange Resins. Dowex-50 resin, 200 to 400 mesh, 8% cross-linked, was treated with 6 N H_2SO_4 until the optical density of the wash liquid measured at 160 mμ was less than 0.025. The resin was equilibrated with 1 N H_2SO_4 prior to use. Dowex-1 resin, 200 to 400 mesh, 10% cross-linked, was converted to the formate form by the method of Cohn [4] Prior to use, the resin was thoroughly washed with $HCOONH_4$ buffer (0.01 M formate, pH 10).

Analytical Methods. Sulfur in the thioether linkage was measured quantitatively by the method of McCarthy and Sullivan,[5] amino nitrogen by the method of Yemm and Cocking,[6] and pentose by the orcinol reaction [7] The paper chromatographic techniques used were described in earlier reports from this laboratory.[8,9]

Results

Preparation of S-Adenosyl-L-homocysteine. S-Adenosyl-L-homocysteine was prepared enzymatically as follows: 1.5 mmoles of adenosine, 6.0 mmoles DL-homocysteine (free base) and 1.0 mmole phosphate buffer (pH 6.5) were diluted to a volume of 100 ml in a 125-ml flask. The use of DL rather than L-homocysteine was more economical Only the L-component reacts[3] and no obvious interference of the D-isomer was noted. The flask was flushed with nitrogen for 10 minutes to produce anaerobic conditions. Five ml of the crude rat liver homogenate was added by syringe, and the reaction mixture was incubated for 3 hours at 37°C. After incubation 0.025 ml of thiodiglycol was added to the reaction mixture to maintain S-adenosyl-L-homocysteine in the reduced state. The reaction mixture was deproteinized by heating in a boiling water bath for 5 minutes. Such heating was also sufficient to destroy small quantities of S-adenosyl-L-methionine introduced with the liver homogenate. The reaction mixture was cooled in an ice bath, and the precipitate was removed by centrifugation at 9000 x g for 20 minutes.

S-Adenosyl-L-homocysteine was purified from the crude reaction material by column chromatography employing Dowex-50 resin (H^+ form). To establish the yield of S-adenosyl-L-homocysteine, 2.0 ml of the reaction mixture was added to a small analytical column and development was begun with 1 N H_2SO_4 (Figure 35). Elution was continued until the optical density of the effluent was less than 0.025 at 260 mμ. Undesired nucleotides, nucleosides, bases and amino acids appeared in this fraction. After passage of approximately 200 ml of 3 N acid through the column, most of the adenine was eluted. Toward the end of this phase S-adenosyl-L-homocysteine began to appear in the effluent. To increase the speed of elution, 6 N acid was added. Typical yields in the 6 N acid fraction, measured by ultraviolet absorption at 260 mμ ($E_m = 16 \times 10^3$), approximated 50% based on the amount of adenosine added to the reaction mixture.

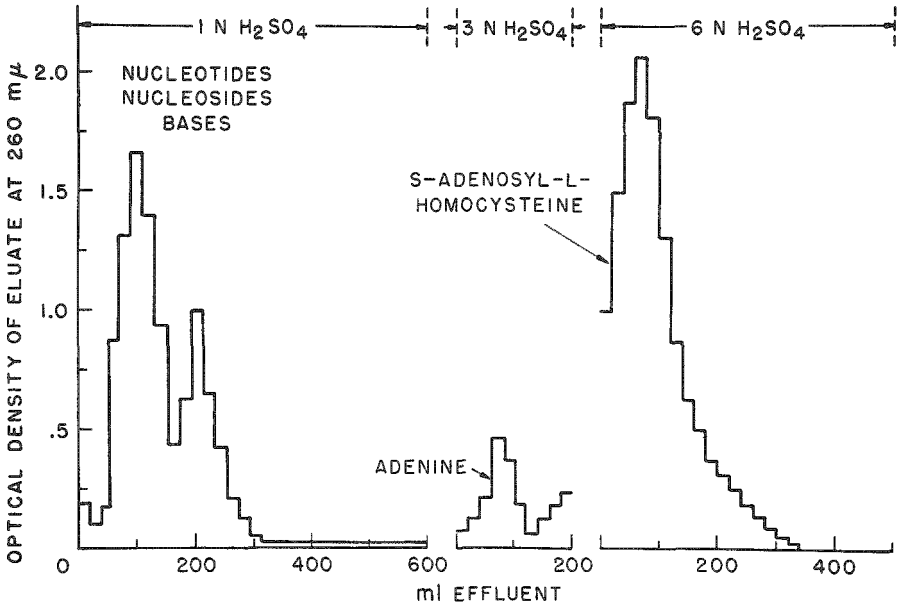

Figure 35. Purification of S-adenosyl-L-homocysteine on Dowex-50 resin; (H^+ form) 7 cm x 0.5 sq cm; flow rate 1.0 ml/minute.

A column 15 cm^2 in cross-sectional area with a resin bed 7 to 10 cm deep was sufficient to purify 100 ml of the reaction mixture. As a precaution against oxidation of S-adenosyl-L-homocysteine, 0.25 ml of thiodiglycol per liter was added to the developing solvents. From 3 to 4 liters of 1 N H_2SO_4 were required to remove most of the undesired compounds and the addition of about 1 liter of 3N acid removed traces of adenine. Finally about 3 liters of 6 N acid eluted S-adenosyl-L-homocysteine from such a column. All column operations were carried out in a cold room maintained at 4°C.

S-Adenosyl-L-homocysteine was precipitated from the 6 N acid eluate by the addition of 20 ml of a 20% phosphotungstic acid solution for each 100 μmoles of compound. The precipitation of S-adenosyl-L-homocysteine was greatly facilitated by the advance addition of the estimated amount of phosphotungstic acid to the collecting flask; occasional agitation was required. The precipitate was allowed to settle overnight in a cold room. Most of the supernatant fluid could be removed by decantation; the remaining fluid was removed by centrifugation at 5000 x g for 10 minutes. The phosphotungstate was washed with 10 volumes of cold distilled water and dissolved in 5 to 6 volumes of acetone-water mixture (1:1) to yield a clear solution. After treatment with the organic solvents to decompose the phosphotungstate[8] the aqueous solution was neutralized to pH 6.5 with a freshly prepared solution of $BaCO_3$. The precipitate was removed by filtration and washed with a small amount of water; the wash liquid was combined with the filtrate. Recovery based on ultraviolet absorption amounted to 35 to 45% of the S-adenosyl-L-homocysteine present in the 6 N eluate. The aqueous solution was

lyophilized and dissolved in water to give a concentration of 45 to 50 μmoles per ml. This solution was filtered to remove undissolved material and the filtrate was frozen and stored at 4°C for 3 to 4 days to complete crystallization. The crystals were collected by filtration, washed with a small amount of cold water, and stored under vacuum over silica gel at -10°C until used.

Graded samples of S-adenosyl-L-homocysteine ranging from 0.04 to 0.4 μmoles were analyzed by chromatography on Whatman No. 1 paper using butanol-water-acetic acid (12:5:3) and ethanol-water-acetic acid (65:34:1) to check for ultraviolet-absorbing and ninhydrin-positive impurities. In all instances S-adenosyl-L-homocysteine was the only compound found. As judged by spectrophotometric measurements at 260 mμ, the crystalline compound was 99% pure.

Preparation of S-Adenosyl-L-homocysteine Sulfoxide. If thiodiglycol was omitted from the eluting acids in the preparation of S-adenosyl-L-homocysteine some oxidation of the latter to S-adenosyl-L-homocysteine sulfoxide occurred. For separation of mixtures of the two compounds, Dowex-1 resin ($HCOO^-$ form) was used. The column was developed as indicated in Figure 36. Two distinct peaks were obtained. The fractions from each peak were pooled and lyophilized; the resultant dry powders were subjected to sublimation[10] at low temperature to remove ammonium formate. In ascending paper chromatography with butanol-water-acetic acid, compound A (isolated from peak 1) showed an R_f value of 0.10, compound B (isolated from peak 2) an R_f of 0.16. Both compounds responded to ultraviolet light scanning and ninhydrin reagent, but only compound B reacted with iodoplatinate reagent. This indicated that the thioether linkage in compound A had been altered.

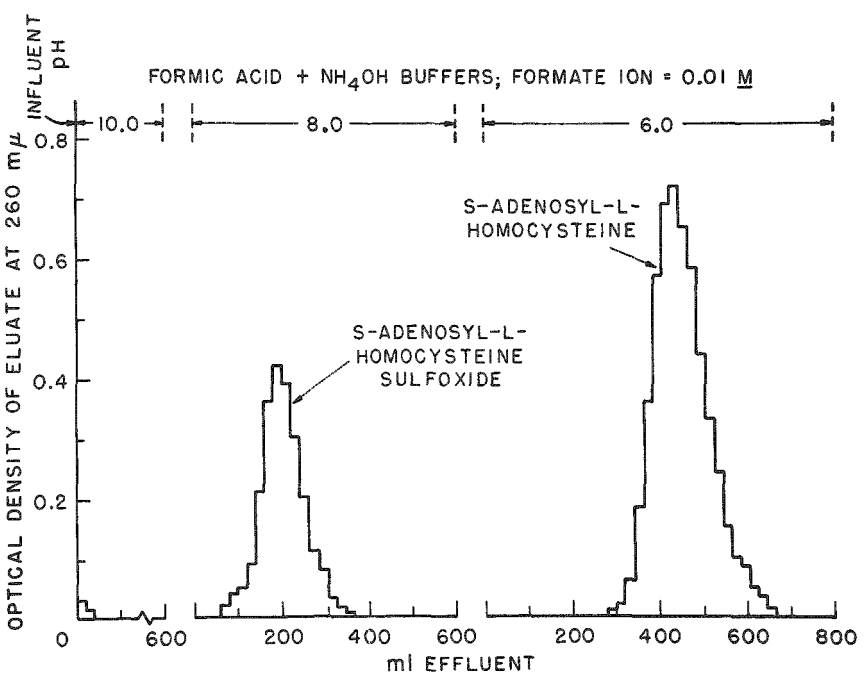

Figure 36

Separation of S-adenosyl-L-homocysteine from S-adenosyl-L-homocysteine sulfoxide; Dowex-1 resin; ($HCOO^-$ form) 15 cm x 0.5 sq cm, flow rate 1.0 ml/minute.

S-Adenosyl-L-homocysteine sulfoxide was prepared by oxidation of S-adenosyl-L-homocysteine by exposure to a 1% solution of H_2O_2 at room temperature for 1 hr. The sulfoxide showed an elution pattern from the Dowex-1 column ($HCOO^-$ form) and migration on paper identical with that of compound A.

Preparation of S-Ribosyl-L-homocysteine. When samples of S-adenosyl-L-homocysteine were analyzed by paper chromatography before crystallization two impurities were noted. One of these was identified as adenine; the other compound reacted with ninhydrin and iodoplatinate spray reagent but did not absorb ultraviolet light. Inasmuch as these compounds could not be detected in the crude reaction mixture, decomposition of S-adenosyl-L-homocysteine during column purification was indicated. Further work showed that decomposition occurred when pure crystalline S-adenosyl-L-homocysteine was placed in 1 or 3 N acid at room temperature for 24 hours. The rate of decomposition was markedly reduced at 4°C.

To obtain more information on the decomposition products of S-adenosyl-L-homocysteine, the pure compound was subjected to weak acid and alkaline hydrolysis. It was found that 0.1 N HCl split the glycosidic bond to yield adenine and a second compound, which was tentatively identified as ribosyl-L-homocysteine by paper chromatography (R_f, 0.44) with ethanol-water-acetic acid, and butanol-water-acetic acid (R_f, 0.14). It did not absorb ultraviolet light, but reacted with iodoplatinate spray reagent, ninhydrin reagent, and carbohydrate reagents such as p-anisidine HCl, ammoniacal silver nitrate, and ammonium molybdate-ammonium chloride.[11] The hydrolysis may be described as follows:

$$\text{S-Adenosyl-L-homocysteine} \xrightarrow[100°C,\ 90\ \text{min}]{0.1\ N\ HCl} \text{adenine} + \text{S-ribosyl-L-homocysteine}$$

To establish further the identity of S-ribosyl-L-homocysteine, sufficient quantities of S-adenosyl-L-homocysteine were hydrolyzed with weak acid, and the hydrolysate was neutralized with NaOH. Purification of S-ribosyl-L-homocysteine was accomplished by paper chromatography. The hydrolysate was spread in a narrow band on Whatman 3-mm paper; with repeated application, as much as 200 μmoles could be placed on a single sheet. After development with the ethanol-water-acetic acid solvent system, the band corresponding to S-ribosyl-L-homocysteine was removed and eluted with water. The eluate was lyophilized and dissolved in water to give a concentration of 10 μmoles per ml. All attempts to crystallize S-ribosyl-L-homocysteine from aqueous solution with or without the aid of various organic solvents failed. The aqueous solution was subjected to various quantitative chemical tests to determine the reactive groups of the molecule. It responded to the orcinol and ninhydrin reagents and reacted as a thioether in the McCarthy-Sullivan reaction, giving an absorption maximum at 505 mμ,

as compared with a peak at 510 mμ for methionine and 500 mμ for S-adenosyl-L-homocysteine. The data given in Table 15 show satisfactory agreement among these tests, but the values are lower than expected from the weight of the lyophilized material used, assuming molecular weight of 267. Apparently the material contained moisture or impurities extracted from the paper. However, the fact that the compound was derived from S-adenosyl-L-homocysteine and possessed the functional groups indicated by the tests (Table 15) suggests the following structure:

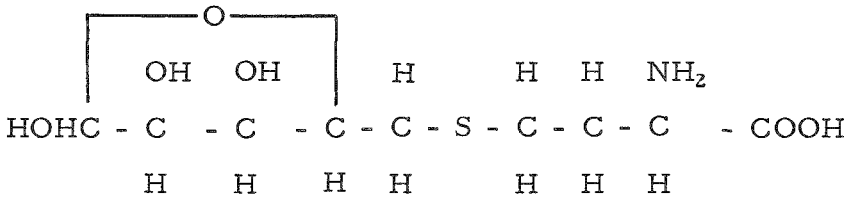

TABLE 15

Analysis of S-Ribosyl-L-homocysteine*

	μmoles/ml
Pentose (Orcinol reaction)	8.70
Amino nitrogen (Quantitative ninhydrin)	8.85
Sulfur (Nitroprusside test)	8.74

*The material was purified by paper chromatography, and a slight correction for impurities derived from the paper was necessary. The reference standards employed were ribose, homocysteine and methionine. The stock solution contained 10 μmoles of material per ml.

In accord with the above structure was the observation that S-ribosyl-L-homocysteine undergoes cleavage with 0.5 N acid to yield homocysteine and ribose:

$$\text{S-Ribosyl-L-homocysteine} \xrightarrow[100°C,\ 90\ \text{min}]{0.5\ \underline{N}\ HCl} \text{ribose} + \text{homocysteine} \quad .$$

S-Ribosyl-L-homocysteine Sulfoxide. S-Adenosyl-L-homocysteine did not undergo appreciable decomposition when subjected to weak alkaline treatment. However, S-adenosyl-L-homocysteine sulfoxide did exhibit considerable breakdown when hydrolyzed with 0.1 N NaOH at 100°C for 90 minutes. Paper chromatography revealed the presence of adenine, homocysteine, ribose and a compound with R_f 0.09 in butanol-water-acetic

acid, and R_f 0.36 in ethanol-water-acetic acid. This material, which reacted with the carbohydrate and ninhydrin spray reagents but not with iodoplatinate, appears to be S-ribosyl-L-homocysteine sulfoxide. Support for this tentative identification was obtained by oxidizing S-ribosyl-L-homocysteine with H_2O_2, as described for the oxidation of S-adenosyl-L-homocysteine to S-adenosyl-L-homocysteine sulfoxide. This oxidation product proved identical with the unknown material obtained from alkaline hydrolysis of S-adenosyl-L-homocysteine sulfoxide.

Discussion

Various modifications in the procedure of de la Haba and Cantoni[3] for the preparation of S-adenosyl-L-homocysteine lead to a pure crystalline compound. The preparation is simplified by the use of a crude rat liver homogenate rather than a partially purified enzyme. Improvement in the isolation was achieved by column chromatography followed by phosphotungstic acid precipitation. Amounts of S-adenosyl-L-homocysteine sufficient for biological studies can be obtained with ease. The final yield of about 20% of the theoretical value is satisfactory since the starting compounds, adenosine and DL-homocysteine, are relatively inexpensive.

Of interest was the finding that S-adenosyl-L-homocysteine underwent considerable oxidation during purification unless a suitable reducing reagent such as thiodiglycol was included. Oxidation of the crystalline compound also occurred unless it was stored in vacuo or in the absence of oxygen.

The results of chemical degradation studies of pure S-adenosyl-L-homocysteine demonstrate the compound to be quite stable to weak alkaline treatment. This is in contrast to the lability of S-adenosyl-L-methionine under similar conditions.[9] On the other hand, S-adenosyl-L-homocysteine undergoes cleavage when subjected to weak acid hydrolysis. Such cleavage leads to the formation of adenine and a new compound which was isolated and partially purified by paper chromatography using several solvent systems. Quantitative tests for the thioether linkage, amino nitrogen and pentose sugar suggest its identification as S-ribosyl-L-homocysteine.

The sulfoxide of S-adenosyl-L-homocysteine has also been prepared. It can easily be separated from the reduced compound by chromatography on Dowex-1 resin $HCOO^-$. The sulfoxide behaves much like S-adenosyl-L-methionine. It has the same R_f values in the solvent systems used and is quite labile to alkaline hydrolysis. This is in accord with the observation that sulfoxides often resemble sulfonium compounds in their chemical properties.

The characterization of S-adenosyl-L-homocysteine sulfoxide, S-ribosyl-L-homocysteine and S-ribosyl-L-homocysteine sulfoxide deserves more than academic interest. It is highly probable that these compounds are intermediates of biological significance.

Summary

A simple procedure for the preparation of crystalline S-adenosyl-L-homocysteine is reported. The product is free from all impurities detectable by conventional methods. S-Adenosyl-L-homocysteine was found to be quite resistant to alkaline treatment; however, it undergoes cleavage in the presence of weak acid, producing adenine and a new compound, S-ribosyl-L-homocysteine. The latter is split with stronger acid to ribose and homocysteine. The sulfoxide of S-adenosyl-L-homocysteine has been prepared. As expected, it is more labile to alkaline treatment than S-adenosyl-L-homocysteine, and the fragments formed are adenine, S-ribosyl-L-homocysteine sulfoxide, homocysteine and ribose.

References

1. Cantoni, G. L., and E. Scarano. The formation of S-adenosyl-L-homocysteine in enzymatic transmethylation reactions. J. Am. Chem. Soc. 76 4744-4749 (1954).

2. Baddiley, J., and G. A. Jamieson. Synthesis of (5'-deoxyadenosine-5')-homocysteine, a product from enzymatic methylations involving "active methionine." J. Chem. Soc. 1085-1089 (1955).

3. de la Haba, G., and G. L. Cantoni. The enzymatic synthesis of S-adenosyl-L-homocysteine from adenosine and homocysteine. J. Biol. Chem. 234 603-608 (1959).

4. Cohn, W. E. The anion-exchange separation of ribonucleotides. J. Am. Chem. Soc. 72 1471-1478 (1950).

5. McCarthy, T. E., and M. X. Sullivan. A new and highly specific test for methionine. J. Biol. Chem. 141 871-876 (1941).

6. Yemm, E. W., and E. C. Cocking. The determination of amino acids with ninhydrin. Analyst 80 209-213 (1955).

7. Brown, A. H. Determination of pentose in the presence of large quantities of glucose. Arch. Biochem. 11 269-278 (1946).

8. Schlenk, F., and R. E. DePalma. The formation of S-adenosylmethionine in yeast. J. Biol. Chem. 229 1037-1050 (1957).

9. Schlenk, F., S. K. Shapiro, and L. W. Parks. Sulfonium compounds and group transfer, especially transmethylation. In Proceedings of International Symposium on Enzyme Chemistry, Tokyo and Kyoto. Tokyo: Muruzen Co., Ltd., 1958. pp. 177-180.

10. Hirs, C. H. W., S. Moore, and W. H. Stein. Isolation of amino acids by chromatography on ion exchange columns, use of volatile buffers. J. Biol. Chem. 195 669-683 (1952).

11. Blosk, R. J., E. L. Durrum, and G. Zweig. A Manual of Paper Chromatography and Paper Electrophoresis, 2nd Ed. New York: Academic Press, Inc., 1958.

STABILIZATION AND AUGMENTATION OF THE NEUTRAL DEOXYRIBONUCLEASE (DNAse I) IN MAMMALIAN TISSUE

R. N. Feinstein and Ulrich Hagen*

Although a form of DNAse optimally active at about pH 5 (DNAse II) has been observed in a variety of mammalian tissues, the form of the enzyme optimally active at about pH 7 (DNAse I) can normally be detected only in pancreas. All other tissues tested contain a considerable excess of an inhibitor of DNAse I.[1] It has not yet proven possible to separate the DNAse I and its inhibitor, nor completely and selectively to destroy either factor in order to measure the other.

The possibility was considered that pancreas is unique in exhibiting DNAse I activity because its high contant of proteolytic enzymes destroys its DNAse inhibitor. If this were so, it might be possible to incubate pancreas with other tissues, e.g., liver, known to have an excess of inhibitor, to the end that the pancreatic proteases would also destroy the liver DNAse I inhibitor. When this experiment was tried, it was found that such mixtures did indeed increase in total DNAse I activity, but it was impossible to decide whether the increased activity originated in the pancreas or the liver. The following experiments were, therefore, performed to clarify this point and to attempt accurate assays of enzyme and inhibitor in a variety of tissues.

Experimental

DNAse I assays were performed by the viscosimetric technique; in all cases, the DNAse was maximally activated with peptone.[1] Beef pancreas was obtained "warm" from the slaughterhouse floor, transported to the laboratory in dry ice, and stored under liquid nitrogen. Less frigid storage results in detectable, but erratic, changes in activity. Crystalline DNAse (once crystallized; originally obtained from beef pancreas) was purchased from Nutritional Biochemicals Corporation. It was dissolved in cold water at 5 μg/ml and stored frozen in individual plastic bottles of 20 to 30 ml capacity. Although a certain loss in activity is sustained during the process of freezing, further loss in activity is minor during several months of storage at -10 to -15°C.

Beef pancreas was blended with water and centrifuged at 55,000 x g in the cold. The supernatant was mixed with varying amounts of egg albumin, and the mixtures were assayed after different periods of incubation at 37°C. Figures 37 and 38 show that the presence of albumin permits the appearance of a considerably augmented DNAse I activity from the pancreas. In one experiment the albumin-free pancreas extract initially increased in activity, then decreased; in the other experiment, no initial increase was observed. We believe that this is due to conditions of storage, as noted earlier. In the

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second experiment the pancreas had been stored several weeks in the freezing unit of the refrigerator at -10 to -15°C.

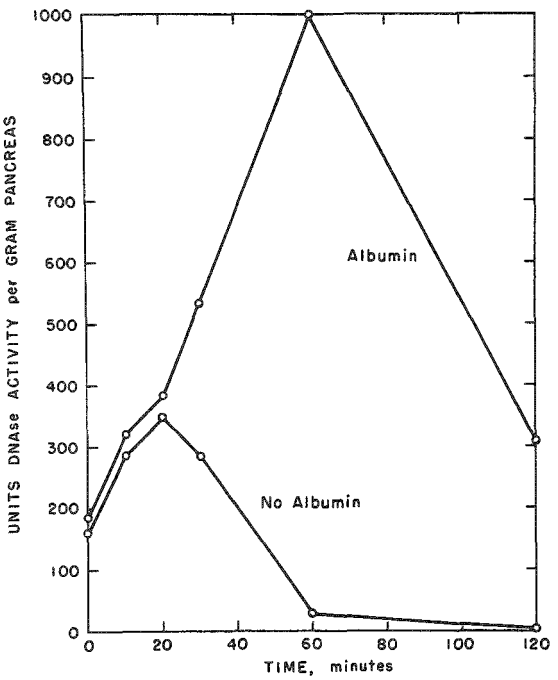

Figure 37

Effect of ovalbumin on DNAse activity of aqueous pancreas extract. Mixture of upper curve contained 0.05% ovalbumin; that of lower curve, no albumin. Incubation was at 37°C.

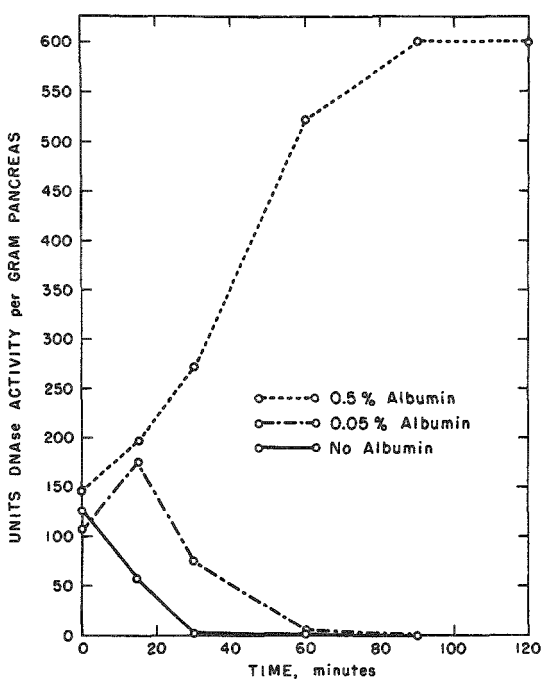

Figure 38

Effect of ovalbumin on DNAse activity of aqueous pancreas extract. Incubation was at 37°C.

Table 16 indicates that the preserving and augmenting effect of egg albumin on pancreatic DNAse is not unique in ovalbumin but extends also to a variety of other proteolytic substrates.

An experiment was performed to determine approximately how long such protective effect would last. In this case, the pancreas was blended with 19 volumes of cold 1% Igepal CO 630, a non-ionic detergent, and centrifuged at 5000 x g; the supernatant was incubated at 37° C with albumin (final

concentration 0.5%) and a few crystals of thymol as preservative. It will be observed from Figure 39 that under these circumstances the DNAse activity remains maximal for at least 36 hr.

TABLE 16

Effect of various protective agents on DNAse activity of pancreas extract

Added	DNAse, units/g	
	Initial	2 Hr
None	222	0
8.0% Peptone	280	834
0.8% Gelatine	176	714
0.8% Albumin	164	690
0.8% Hemoglobin	170	446
Boiled liver supernatant	186	164

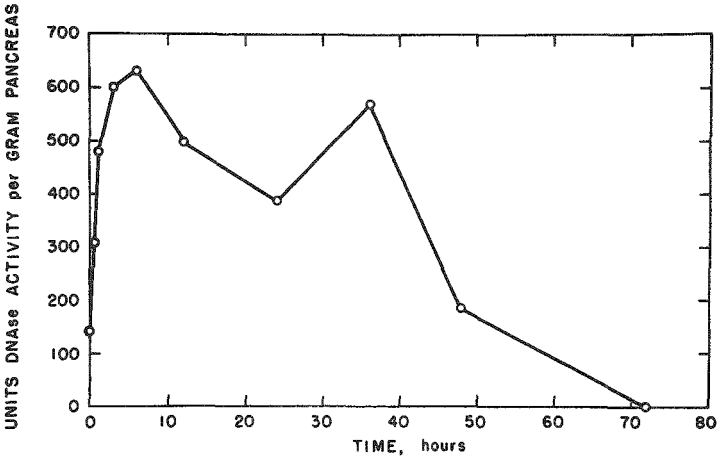

Figure 39

Duration of albumin protection on DNAse activity of pancreas extracts in non-ionic detergent. Ovalbumin was initially present at a concentration in the mixture of 0.5%; thymol was added as a preservative.

The albumin device was also used in an attempt to demonstrate DNAse I activity in other tissues. Mouse liver, kidney, and intestine were homogenized in 9 volumes of cold 1% Igepal CO 630 and centrifuged and incubated as above. The function of the Igepal is twofold: (a) it solubilizes more tissue protein;[2] (b) and it apparently helps destroy or dissociate DNAse inhibitor.[3] Figure 40 shows that DNAse I activity, not initially apparent in these tissues, becomes obvious in intestine and kidney within 30 to 60 min and maximal at about 36 hr. It should be noted, however, that the scale of activity in this figure differs from that in Figure 39. Per gram of tissue, the maximal pancreatic activity is about 200 times that of kidney or intestine.

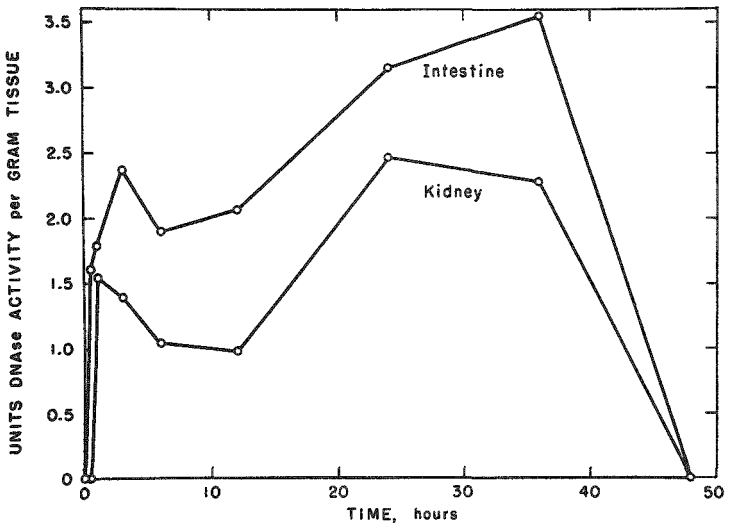

Figure 40

Duration of ovalbumin effect on DNAse activity of detergent extracts of mouse kidney and intestine. Ovalbumin was originally present at a concentration in the mixture of 0.5%; thymol was added as a preservative.

Albumin also has the effect of preserving solutions of crystalline DNAse from loss of activity upon incubating at 37°C. Two such experiments are briefly summarized in Table 17, which indicates that the customary deterioration of the enzyme at 37°C is eliminated by the presence of albumin.

TABLE 17

Effect of albumin on crystalline DNAse incubating at 37°C

		DNAse, units/g	
		Initial	1 hr
Experiment 1	Control	0.9	0.4
	Albumin	0.8	0.8
Experiment 2	Control	1.2	0.5
	Albumin	1.4	1.4

The possibility was considered that this might also be a protective action of albumin against trace contamination of the crystalline DNAse with proteolytic activity. This point was investigated by incubating the crystalline enzyme with varying amounts of albumin. If the albumin were protecting against proteolysis, it would be expected that at some concentration of albumin, protection would be evident for a period of time, and then, upon the proteolysis of the albumin, destruction of the DNAse would ensue. Figure 41 shows that no such concentration of albumin could be found, and it is concluded that degradation of these dilute solutions of crystalline DNAse is via denaturation.

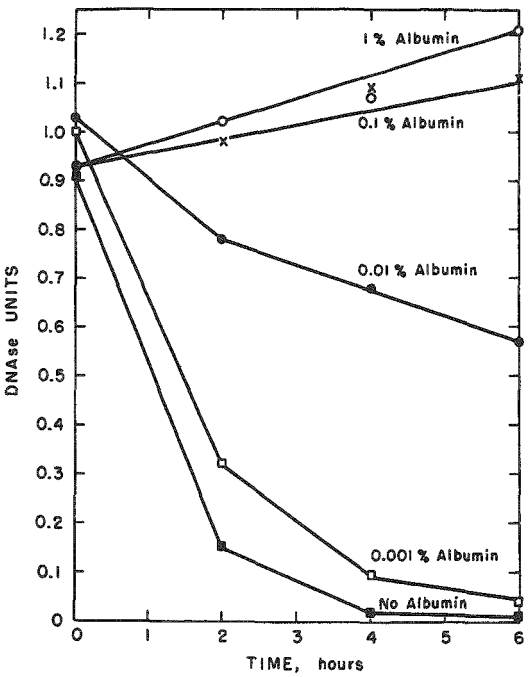

Figure 41

Effect of varying quantity of ovalbumin on activity of aqueous solution of crystalline DNAse. Incubation was at 37°C.

An experiment was also performed to decide whether the presumed complex formed by the admixture of crystalline DNAse and liver supernatant was a dissociable one. To this end, a solution of crystalline DNAse and a mixture of the same amount of crystalline DNAse together with liver supernatant were prepared, and these two solutions were serially diluted and then assayed. Table 18 indicates that the greater the dilution of these solutions, the less inhibition is evident. It is, therefore, concluded that this enzyme-inhibitor complex is indeed dissociable.

TABLE 18

Dissociability of the DNAse-inhibitor complex

Relative concentration of solution	DNAse I activity, units/g		% inhibition
	No inhibitor	Inhibitor	
100	0.56	0.30	46
75	0.47	0.25	47
50	0.32	0.23	28
30	0.18	0.16	11
15	0.10	0.105	0

Discussion

A primary goal of this work has been the attainment of a technique for liberating and measuring the total DNAse I activity of other tissues than pancreas. Although the rather prolonged constancy of the kidney and intestine DNAse levels shown in Figure 40 suggest that maximum activity may have been reached, there is some reason to question this conclusion. Particularly to be noted is the fact that liver here showed only traces of activity, whereas we,[3] as well as others,[4-8] have demonstrated appreciable liver DNAse I activity under various other conditions.

The mechanism whereby albumin not only protects the DNAse I of pancreas, but actually permits an augmented activity, would seem most readily explainable by the concept that the pancreatic inhibitor of DNAse activity is most labile to pancreatic proteases, that the DNAse itself is less sensitive, and that ovalbumin is of an intermediate stability. Thus upon incubation the inhibitor is destroyed, while the enzyme is protected by saturation of the proteases with albumin. It has been suggested before[5] that tissue DNAse may be more stable to proteolytic activity than is the inhibitor.

Summary

Aqueous extracts of beef pancreas rapidly lose their DNAse I activity upon incubation at 37°C. If albumin, peptone, or a variety of other proteolytic substrates is added to the extract, not only is there no loss of activity, but a severalfold increase in activity is observed. Mouse kidney or intestine, which normally show no DNAse I activity, will exhibit an appreciable, though not great, activity if similarly incubated with albumin.

Once-crystallized commercial DNAse also loses activity upon incubation, but not in the presence of albumin or peptone. Protection against loss of activity in this system is presumably due to protection against denaturation. In the case of crude pancreas extracts, the protection is presumed due to proteolytic destruction of DNAse inhibitor while DNAse itself is protected from proteolysis.

The complex formed between crystalline DNAse and DNAse inhibitor from mouse liver is dissociable upon dilution.

References

1. Feinstein, R. N. Activation of the neutral deoxyribonuclease. J. Biol. Chem. 235 733-737 (1960).

2. Feinstein, R. N. Solubilization of particulate catalase with nonionic detergent. Arch. Biochem. Biophys. 79 399-400 (1959).

3. Feinstein, R. N., and U. Hagen. Tissue distribution of the neutral deoxyribonuclease (DNAse I). Fed. Proc. 18 225 (1959).

4. Baudhuin, P. Localisation intracellulaire et activation des désoxyribonucléases du foie de rat. Arch. Intern. Physiol. 67 106-107 (1959).

5. Cooper, E. J., M. L. Trautman, and M. Laskowski. Occurrence and distribution of an inhibitor for desoxyribonuclease in animal tissues. Proc. Soc. Exptl. Biol. Med 73 219 (1950).

6. Shack, J. Deoxyribonucleases of mouse tissues. J. Biol. Chem. 226 573-581 (1957).

7. Siebert, G., K. Lang, and A. Corbet. Über das pH-Optimum der Desoxyribonuclease verschiedener Tierarten. Biochem. Z. 320 418-421 (1950).

8. Siebert, G., K. Lang, S. Lucius-Lang, L. Herbert, G. Stark, G. Rossmüller, and H. Jöckel. Eigenschaften und biologische Bedeutung der Desoxyribonuclease aus Säugetierorganen. Z. physiol. Chem. 295 229-243 (1953).

S-ADENOSYLMETHIONINE IN RAT LIVER

Panpit Pansuwana*

Despite extensive experiments on the formation of S-adenosylmethionine by liver enzymes and on its function as a methyl donor in the formation of creatine, N-methylnicotinamide, choline, and other methylated compounds, no information is on record concerning the concentration of this sulfonium compound in tissues.[1] In the mammalian organism liver is recognized as the principal site of formation and function of S-adenosylmethionine; therefore, its quantitative determination in this tissue and the correlation of the concentration with the amounts of methylated products is of particular interest.

In the past, an obstacle to tissue assay has been the lack of suitable methods. This difficulty has been overcome by the development of simple chromatographic techniques combined with spectrophotometry, taking advantage of the strongly cationic properties of the sulfonium compound and of its ultraviolet absorption at 260 mμ.[2] With yeast cells it has been demonstrated that the methionine concentration is a limiting factor in the formation of S-adenosylmethionine.[2] A similar situation may be expected in mammalian liver since methionine is an indispensable dietary constituent. Experiments on the influence of methionine administration on the formation of S-adenosylmethionine were included, therefore, in this series. The use of labeled methionine gave some information on the turnover of S-adenosylmethionine.

Materials and Methods

Rats (Sprague-Dawley) of either sex, weighing approximately 200 g, were used. Immediately after death the livers were homogenized with 2 or 3 volumes of distilled water at low temperature. For extraction and removal of protein, perchloric acid was added to the homogenate[3] to a final concentration of 1.5 N. The mixture was kept in the cold room with frequent agitation for two hours. After centrifugation, the supernatant solution was applied to a Dowex 50 H^+ column as described earlier.[2] Nucleotides and related substances were eluted by 1 N H_2SO_4; S-adenosylmethionine was released from the column by increasing the concentration of the acid to 6 N. The concentration of S-adenosylmethionine in the eluate was determined by spectrophotometry at 256 mμ (E_m = 15,400). The radioactivity of samples was determined by liquid scintillation counting.

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*On leave of absence from Siriraj Hospital, Dhonburi, Thailand; sponsored by the International Institute of Nuclear Science and Engineering.

Results and Discussion

Table 19 shows data on the concentration of S-adenosylmethionine in rat liver. The level is much lower than that in microorganisms, especially in yeast.[1] Furthermore, the concentration varies considerably among animals and with the state of nutrition. The influence of L-methionine was tested by intraperitoneal injection at different times prior to sacrifice of the animals. The effect of the amino acid in raising the level of S-adenosylmethionine in liver apparently has reached its maximum after 30 min. The increase, however, is small compared with the amount of methionine injected. It must be concluded that the level of methionine in the tissue is only one of several factors determining the concentration of S-adenosylmethionine, and the compound appears to be metabolized and eliminated rapidly. At 2 hr and at 4 hr after intraperitoneal injection of S-adenosylmethione, normal levels were found in liver.

TABLE 19

S-Adenosylmethionine in rat liver

Treatment	Interval before sacrifice	No. of animals	Liver S-adenosylmethionine, μM/g
Food ad libitum		13	0.012-0.136 (mean 0.075)
Starved	24 hr	4	0.01-0.09 (mean 0.04)
L-methionine* (67 μM)	15 min	1	0.11
	30 min	2	0.20
	2 hr	2	0.10
	4 hr	2	0.07
L-methionine* (200 μM)	30 min	1	0.45
S-adenosylmethionine* (50 μM)	2 hr	1	0.06
	4 hr	1	0.05

*Intraperitoneal injection.

Tracer experiments were carried out to check for the presence of methionine reserves in liver. This may be judged on the basis of dilution of the specific activity of S-adenosylmethionine after injection of the labeled amino acid. Table 20 shows that considerable dilution occurs which can be accounted for only in part by the nonradioactive S-adenosylmethionine present in the liver at the outset of the experiment. The discrepancy

between methionine-α-C^{14} and methionine-$C^{14}H_3$ in this experiment is explained by the rapid metabolic transfer of the methyl group. S-Adenosylhomocysteine which remains after transmethylation may serve for regeneration of S-adenosylmethionine by adding a methyl group or a methyl precursor. This would explain the greater persistence of the α-C^{14} label compared with that in the methyl group.

TABLE 20

Incorporation of labeled methionine into S-adenosylmethionine in rat liver

Tracer injected*	Quantity, mg	Specific activity, 1×10^5 cpm/μM	Interval before sacrifice, min	Specific activity of liver S-adenosylmethionine, 1×10^4 cpm/μM	% Initial specific activity
L-Methionine-α-C^{14}	32.2	0.79	30	5.5	70
L-Methionine-α-C^{14}	28.4	1.25	120	1.44	11.5
L-Methionine-α-C^{14}	28.4	1.25	240	0.22	1.8
L-Methionine-$C^{14}H_3$	27.2	0.46	30	1.28	28
L-Methionine-$C^{14}H_3$	27.0	0.42	120	0.05	1
L-Methionine-$C^{14}H_3$	27.0	0.42	240	0.05	1

*Intraperitoneal injection. Methionine-α-C^{14} was available only in its DL-form. This material was diluted with an approximately tenfold quantity of nonradioactive L-methionine; only the radioactivity of the L-component is considered in this calculation, since D-methionine does not form S-adenosyl-D-methionine in the animal organism.[4]

The concentration of S-adenosylmethionine in liver is low compared with the quantities of metabolites which are methylated by this donor. It is clear from the present data that the sulfonium compound amounts only to a fraction of the total of methylated products. The content of S-adenosylmethionine per rat liver is usually about 1 μM, while the daily urinary excretion of creatine and creatinine alone is of the order of 40 to 60 μM.[5,6] The biosynthesis of S-adenosylmethionine must be a very effective process to cope with the needs of the organism for methyl groups in this and many other reactions; this is borne out by the rapid disappearance of the label in the tracer experiments.

References

1. Shapiro, S. K., and F. Schlenk. The biochemistry of sulfonium compounds. Advances in Enzymology 22 237-280 (1960).

2. Schlenk, F., and R. E. DePalma. The formation of S-adenosylmethionine in yeast. J. Biol. Chem. 229 1037-1050 (1957).

3. Neuberg, C., E. Strauss, and L. E. Lipkin. Convenient method for deproteinization. Arch. Biochem. Biophys. 4 101-104 (1944).

4. Cantoni, G. L., and P. J. Vignos. Enzymatic mechanism of creatine synthesis. J. Biol. Chem. 209 647-659 (1954).

5. Bodansky, M. The influence of feeding amino acids and other compounds on the excretion of creatine and creatinine. J. Biol. Chem. 112 615-624 (1936).

6. Kumta, U. S., S. U. Gurnani, and M. B. Sanasrabudhe. Influence of post irradiation administration of methionine on the excretion of creatine, creatinine, and N-methyl nicotinamide in urine of rats. Experientia 13 372-373 (1957).

STUDIES ON THE ACID PHOSPHATASE AND β-GLUCURONIDASE ACTIVITIES OF THYMUS AND SPLEEN AFTER WHOLE-BODY X-IRRADIATION OF RATS, AND THEIR POSSIBLE RELATION TO THE LYSOSOME PARTICLES

Y. E. Rahman*

Introduction

On the basis of differential centrifugation, de Duve and co-workers[1,2] proposed the name "lysosome" for a special group of particles which show a sedimentation distribution between the mitochondrial and the microsomal fractions; this group of particles contains a number of easily soluble hydrolases (i.e. acid phosphatase, β-glucuronidase, acid ribonuclease, deoxyribonuclease II, cathepsin, etc.) having in common an acid pH optimum.[3]

In order to study the effect of X-irradiation on this particular group of lysosomes, we have used the thymus and the spleen of rats, since lymphoid tissue is far more radiosensitive than other tissues in which lysosomes have been studied.

Among the lysosomal group of enzymes, so far we have tested only acid phosphatase and β-glucuronidase.

Methods

Two sets of experiments were carried out with female Holtzmann rats, from 45 to 50 days of age, weighing between 130 and 150 g. The first experiment consisted of 32 animals, 24 of which were given a single dose of 200 r; 8 were used as controls. In the second experiment, 48 rats were studied, 36 of which were given a single dose of 1000 r; 12 were used as controls.

Animals were killed by decapitation; thymus and spleen were taken out and chilled at once in 0.25 M sucrose at 0°C; the tissue was passed through a piece of gauze which retained the connective tissue.

Total activites were estimated in the presence of 0.1% Igepal-630, which releases the enzymes completely from the particulate membrane of lysosomes.

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*Resident Research Associate

Free activities were estimated in the presence of a substrate mixture containing 0.25 M sucrose, with an incubation time of ten minutes. The enzymes assays were the same as described by Gianetto and de Duve.(4)

Results

The results are summarized in Tables 21 and 22. From these tables, it is clear that the specific activities of both the acid phosphatase and β-glucuronidase increase after irradiation. However, the total enzyme activities show an increase only in the case of acid phosphatase 2 hr after a dose of 1000 r; this is true for the thymus as well as for the spleen: In all other cases, the total organ activity of the enzymes is decreased. This suggests that the increases in enzyme specific activities may be solely due to a selective retention of enzyme nitrogen.

TABLE 21

Effect of whole-body X-irradiation on the thymus of rats

Time after irradiation	Acid phosphatase			β-glucuronidase		
	200 r					
	E.S.A.	E.T.A.	F.A.	E.S.A.	E.T.A.	F. A.
Control	0.24	3.91	49	5.6	91	47.4
5 hr	0.21	3.44	55	4.5	74	62.5
24 hr	0.66	3.87	68	14.6	86	59.2
48 hr	0.64	2.59	48.4	14.1	57	47.7
	1000 r					
Control	0.15	2.42	65.5	5.6	91	40.0
2 hr	0.19	3.12	64.2	4.9	81	51.2
24 hr	0.20	1.90	73.8	8.4	80	56.5
48 hr	0.64	1.68	53.3	27.7	73	57.6

E.S.A. = Enzyme specific activity, μgP/mgN/min for acid phosphatase, and μ moles of phenolphthalein/gN/min for β-glucuronidase.

E.T.A. = Enzyme total activity product of total nitrogen and E.S.A.

F.A. = Free activity, expressed in % of total activity.

TABLE 22

Effect of whole-body X-irradiation on the spleen of rats

Time after irradiation	Acid phosphatase			β-glucuronidase		
	200 r					
	E.S.A.	E.T.A.	F.A.	E.S.A.	E.T.A.	F.A.
Control	0.38	10.90	53.1	1.5	43	60.0
5 hr	0.31	10.10	55.8	1.4	46	65.0
24 hr	0.56	9.10	50.9	2.2	36	57.0
48 hr	0.69	9.03	52.1	2.4	31	62.5
	1000 r					
Control	0.20	8.62	51.5	1.3	56	44.5
2 hr	0.36	11.90	52.1	1.4	46	54.3
24 hr	0.41	6.65	48.1	2.5	41	54.7
48 hr	0.52	5.38	60.9	3.3	34	61.4

E.S.A. = Enzyme specific activity, μgP/mgN/min for acid phosphatase, and μM of phenolphthalein/gN/min for β-glucuronidase.

E.T.A. = Enzyme total activity, product of total nitrogen and E.S.A.

F.A. = Free activity, expressed in % of total activity.

An attempt at a better understanding of the real significance of the increase of enzyme activities as well as their relation to the lysosome particles has been undertaken by studying the thymus tissue and fractions separated by density-gradient centrifugation under the electron microscope before and after irradiation.

References

1. Appelmans, F., R. Wattiaux, and C. de Duve. Tissue fractionation studies. 5. The association of acid phosphatase with a special class of cytoplasmic granules in rat liver. Biochem. J. 59 438-445 (1955).

2. de Duve, C., B. C. Pressman, R. Gianetto, R. Wattiaux, and F. Appelmans, Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. Biochem. J. 60 604-617 (1955).

3. de Duve, C. In Subcellular Particles. Washington, D.C.: American Physiological Society, 1959. pp. 128-129.

4. Gianetto. R. and C. de Duve. Tissue fractionation studies. 4. Comparative study of the binding of acid phosphatase, β-glucuronidase and cathepsin by rat-liver particles. Biochem. J. 59 433-438 (1955).

THE TEMPERATURE OF NEUTRON-IRRADIATED SUBSTANCES

T. R. Sato and H. H. Strain

When phosphorus compounds were exposed to neutrons at a high flux (2×10^{13} n/cm^2 sec), the activation products varied with the nature of the containers.[1] This variation was traced to thermal reactions resulting from the absorption of neutrons by the small quantities of boron in the containers themselves.

In order to control the condition of the samples during the neutron irradiation, we established the relationship among neutron flux, composition of the containers, and the temperature. To this end, we irradiated ampoules of lime glass, boron-free glass, quartz, and polyethylene using a neutron flux of 1×10^8 (n/cm^2 sec) and determined the temperature as a function of time. Typical results are shown in Figure 42, which indicates that, even at the very low flux, the temperature of lime glass rises significantly while the temperature of the other containers does not increase. For the maintenance of minimum temperature during the neutron irradiation of samples in sealed containers, pure quartz or polyethylene would therefore be the most desirable material.

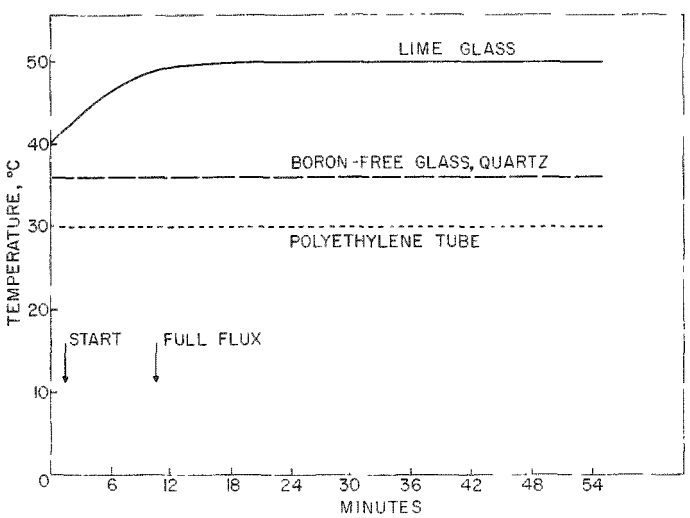

Figure 42. Temperature reached by containers of lime glass, boron-free glass, quartz, and polyethylene irradiated by neutrons at a flux of 1×10^8 n/cm^2 sec. Initial temperatures were those of the reactor before it was brought into operation. The containers were placed in the so-called isotope hole No. 2 in the graphite reflector of the Argonne CP-5 reactor. The temperature was determined with chromel-alumel thermocouples.

The temperature reached by quartz containers at various levels of neutron irradiation is shown by Figure 43. Even with quartz there was a small temperature rise with an increase in the neutron flux, and this should be considered when labile substances are irradiated in sealed quartz tubes.

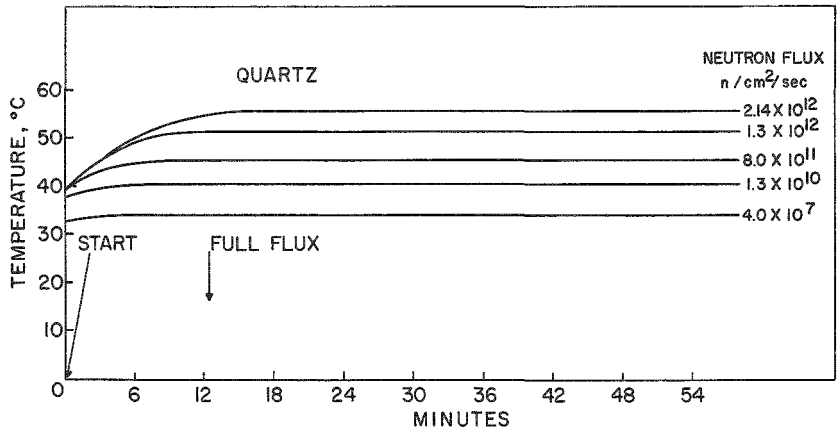

Figure 43. Temperature reached by quartz containers exposed to various levels of neutron flux. The containers were placed in the graphite reflector at various distances from the vertical center line of the reactor where the neutron flux had been determined. Temperature was determined with thermocouples.

Although it is usually desirable to irradiate substances at low temperatures, there may be occasions when an elevated temperature is preferred. By using containers containing boron, as for example Pyrex (borosilicate) glass, elevated temperatures may be achieved. With these containers, the temperature is a function of the neutron flux as shown in Figure 44. These results indicate that it should be possible to prepare containers or packed tubes containing mixtures of boric oxide and alumina so that the temperature depends upon the composition of the mixture; hence for any flux a particular temperature may be obtained.

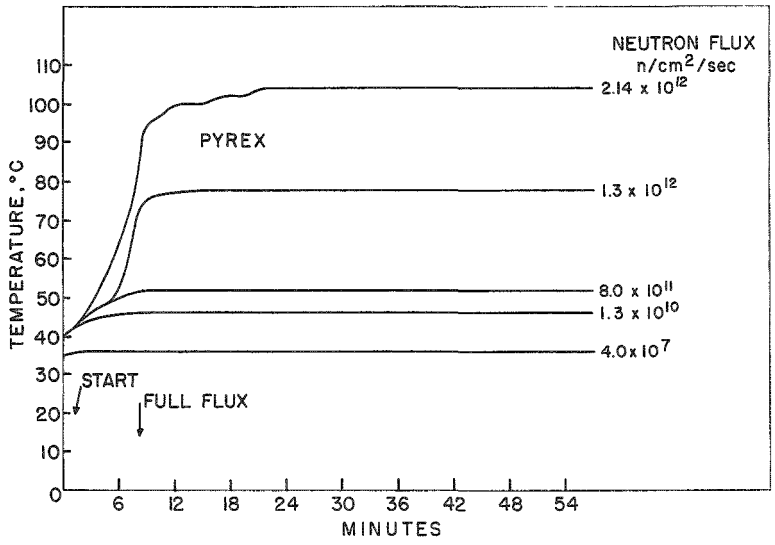

Figure 44. Temperature reached by Pyrex containers exposed to various levels of neutron flux. The conditions were the same as those employed in Figure 43.

The relationship among the composition of the containers, the temperature, and the neutron flux is shown by Figure 45, which is derived from the data in Figures 43 and 44. This figure shows that at a low flux (1×10^7 to 1×10^{11}) the composition of the container has relatively little effect on temperature whereas at high flux (above 1×10^{11}) the composition of the container has a tremendous effect upon the temperature. From this we conclude that for irradiation at low flux (below 1×10^{10}), the temperature effects due to variation of the containers are not very significant. But at high flux (above 1×10^{11}), the temperature effects due to variation of the containers may be critical.

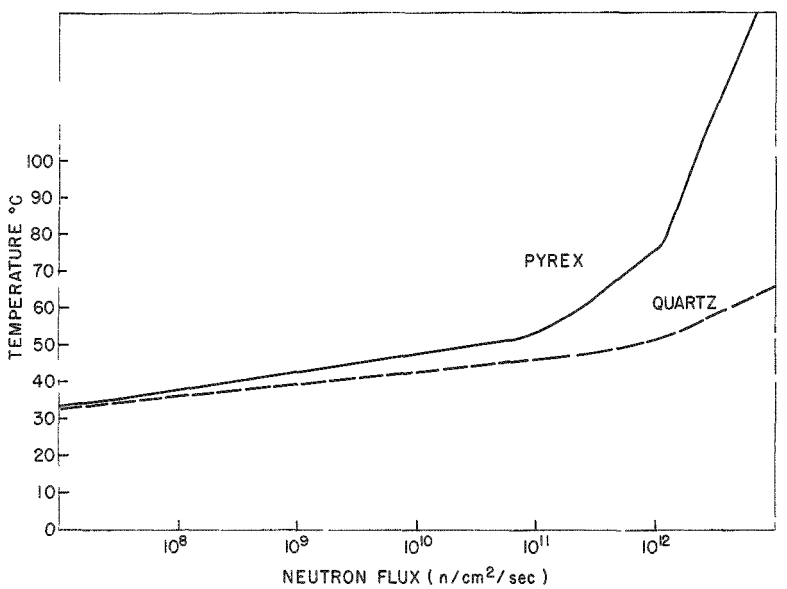

Figure 45. Temperature reached by Pyrex and quartz containers exposed to various levels of neutron flux. Average values were taken from Figures 43 and 44.

We have put these observations to practical use in our studies of the neutron activation of acid phosphates, such as disodium phosphate, Na_2HPO_4. Irradiated at high neutron flux where the temperature of the containers exceeded about 200°C, the entire samples of this material were converted to pyrophosphate, $Na_4P_2O_7$. But irradiated at low neutron flux so that the containers were not heated over 40 to 50°C, the samples were unaltered chemically, yielding only radioactive phosphate, which was isolated by electrochromatography.

Reference

1. Sato, T. R. The production of heat by the neutron irradiation of quartz, boron-free glass and lime glass, and its effect upon the condensation of phosphate. Radiation Research 9 177 (1958).

THE STUDY OF DIURNAL METABOLIC RHYTHMS WITH THE CONSTANT FEEDING TECHNIQUE

I. Liver Glycogen Levels and DNA Synthesis

R. W. Swick, G. Germek and R. Ogawa

The technique of training animals to feed at hourly intervals, the so-called constant-feeding technique, has been used for a number of years in this laboratory for the "continuous" administration of isotopic compounds. The incorporation of tracer materials into the chemical constituents of the rat under these conditions has enabled us to determine certain precursor-product relationships as well as to measure quantitatively the velocities of some metabolic reactions. The question has often been raised whether animals maintained on such an abnormal regimen yield information applicable to normal animals whose feeding habits are considerably different: By changing the rat from a primarily nocturnal feeder to a continuous feeder do we change or distort his metabolism sufficiently to invalidate the experimental results?

From numerous experiments of others, it is clear that not only do animals feed during certain periods of the day and fast at others, but that a number of metabolic processes also exhibit a cyclic pattern of activity. For example, Barnum et al.[1] observed that the stores of liver glycogen in mice maintained under normal light-dark conditions were up to 10 times higher at 8:00 A.M. than they were at 8:00 P.M. These authors also found that the incorporation of P^{32} into microsomal ribose nucleic acid (RNA) and into deoxyribose nucleic acid (DNA) varied over a twofold range during the 24-hr period with each exhibiting a maximum at a different time.

Likewise, Elfvin et al.[2] observed a diurnal rhythm in the glycogen stores of chickens. Because the rhythm seemed to persist in fasting chickens, and a glycogen rhythm of a sort was observed even in chick embryos, the opinion was that the feeding habits of the animal have little or no effect on these cyclic metabolic processes. However, no experimental results have been reported which directly test the point.

Therefore, it appeared feasible to answer, in part, both questions by comparing these metabolic rhythms in animals maintained under normal, ad libitum conditions with those in rats fed continuously. If the constantly-fed rats ceased to exhibit these metabolic cycles, then one might conclude that the rhythms observed in the animals fed ad libitum were a product of the feeding habits of the animal, a secondary response to the immediate nutritional state of the animal, and that the primary rhythms to study are those governing the feeding rhythm. On the other hand, if the cyclic phenomena persist in the continuously fed animal, then these rhythms may well be under the primary control.

Therefore, this report is concerned with a preliminary experiment with only a few animals per group, wherein the liver glycogen levels and the incorporation of P^{32} into various chemical fractions of liver were determined at 6-hr intervals in rats which had been fed ad libitum or hourly. The results are interesting in that constant feeding appears to dampen the cyclic changes in liver glycogen levels but has no effect on the rhythmic changes in the rate of synthesis of DNA.

Twenty-four adult male rats were fasted over a weekend. Of these, 12 were then fed powdered stock diet at hourly intervals for a period of 2 or 3 weeks, a time believed sufficient to bring them into some sort of equilibrium. The rest of the rats were housed in similar cages but were fed the same diet ad libitum. The clock-controlled lighting system gave 12.5 hours of darkness and 11.5 hours of light each day. Three animals in each group were sacrificed at each of 4 time intervals: 7 A.M., 1 P.M., 7 P.M., and 1 A.M. Two hours before sacrifice the animals were injected with $Na_2HP^{32}O_4$. The livers were removed and several slices were taken for mitotic cell counts. The remainder was frozen until it could be analyzed. After thawing, each liver was homogenized; an aliquot was removed for determination of the glycogen, and the remainder was fractionated for isolation of the acid-soluble phosphorus component (ASP), phospholipids, RNA, and DNA. The specific activity of each fraction was determined and compared to the specific activity of the ASP component.

The rats fed ad libitum gained slightly more weight during the equilibration period than did the constant-fed animals, mainly because of the restricted amount of diet offered the latter group during the first few days of the training period. It was not necessary to pair-feed the two groups as both consumed similar amounts of the diet after the proper levels for the constant fed group had become established.

The percentage of glycogen in the livers of the two groups is shown in Figure 46. The glycogen levels in the control animals were highest in those rats killed at 7 A.M. and lowest in those sacrificed at 7 P.M. These results are qualitatively similar to those obtained with mice[1] but quantitatively there is a considerable difference. The values obtained with rats have only about a twofold range, while those observed with mice had roughly a tenfold range. As can be seen from the figure, the glycogen levels at each time period in those animals fed hourly was similar and approximately halfway between the maximum and minimum values for the rats fed ad libitum. Therefore one may conclude that the diurnal variation in liver glycogen levels is a reflection of the feeding habits of the animal. This is not too surprising.

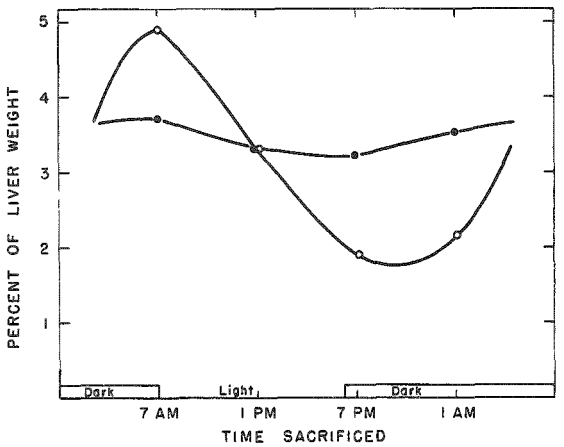

Figure 46

Liver glycogen levels in rats sacrificed at 6-hr time intervals. ○ = animals fed ad libitum; ● = animals fed hourly.

Next, the specific activity of the DNA, RNA and phospholipids for each animal was compared to the specific activity of the ASP fraction obtained from the same animal. The incorporation of P^{32} into the phospholipid fraction did not appear to follow any sort of cyclic pattern in either group; the average for each time group varied about ± 10% of the over-all average, and there was no significant difference between the animals fed ad libitum and hourly. In contrast, in the mouse Barnum et al.[1] found a definite cyclic pattern for this fraction which ranged ± 30% of the average.

The incorporation of isotope into the RNA fraction of liver also failed to show clear-cut differences at different times of the day in animals fed ad libitum or hourly. There was a suggestion of a cyclic pattern in the control animals with a maximum occurring at the end of the light period similar to that found in mice,[1] but the number of animals was too limited to lend much credence to it. In the animals fed hourly, this cycle was even less clear.

Finally the incorporation of P^{32} into the DNA is shown in Figure 47. In both groups DNA synthesis was at its highest at 1 P.M. and at its lowest at 7 P.M. The average values for each group at each time point are quite similar and there is no evidence of any suppression of the cyclic nature of the rate of synthesis in animals fed hourly. In the mouse experiment[1] the maximum occurred near the end of the dark period, following the rise in liver glycogen.

In mice, the mitotic rate in the liver exhibits a fivefold variation through the 24-hr cycle, with a maximum occurring shortly after the peak in DNA synthesis.[1] Through the courtesy of Mrs. A. Stroud, an attempt was made to measure the mitotic rate in the livers of the rats in the present experiment; however, the mitotic rates were so low in all of the animals that it was not possible to make any sort of reasonable count or comparison between groups.

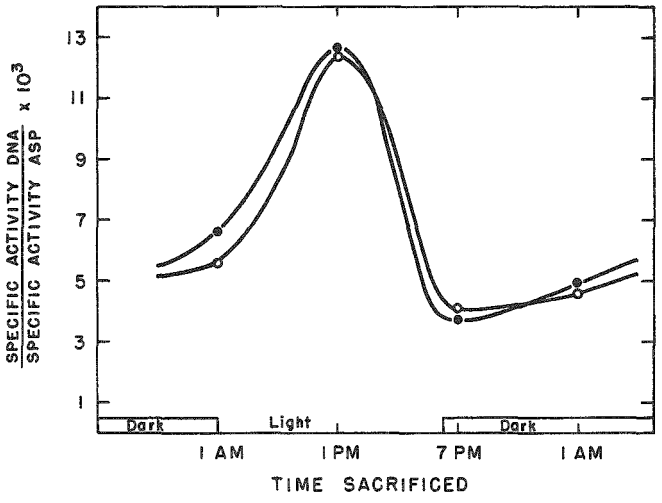

Figure 47. Comparative rates of DNA synthesis in the livers of rats sacrificed at 6-hr intervals. O = animals fed ad libitum; ● = fed hourly.

It appears then that the level of glycogen in the liver may be dependent on the feeding habits of the animal, while the synthesis of DNA as measured by the incorporation of P^{32} is independent of the feeding cycle and is controlled by some more fundamental mechanism. Although the conclusions drawn from the earlier experiments measuring the absolute rates of DNA synthesis in rat liver[3,4] would appear to be undisturbed, the present results suggest that one must at least consider the possible effects of the constant-feeding technique on the metabolic processes of the animal.

Addendum

An attempt was made to repeat the entire experiment with more animals and more time points. The results were quite capricious, and the patterns of the values were only vaguely similar to those obtained in the initial experiment.

References

1. Barnum, C. P., C. D. Jardetzky and F. Halberg. Time relations among metabolic and morphologic 24-hour changes in mouse liver. Am. J. Physiol. 195 301-310 (1958).

2. Elfvin, L. G., T. Petrén and A. Sollberger. Influence of some endogenous and exogenous factors on diurnal glycogen rhythm in chicken. Acta Anat. 25 286-309 (1955)

3. Swick, R. W., A. L. Koch and D. T. Handa. The measurement of nucleic acid turnover in rat liver. Arch. Biochem. Biophys. 63 226-242 (1956).

4. Swick, R. W. and A. L. Koch. The measurement of nucleic acid phosphorus turnover in rat liver by the constant exposure technique. Arch. Biochem. Biophys. 67 59-73 (1957).

PROGRESS REPORT: DENSITY GRADIENT CENTRIFUGATION

Effect of X-Radiation on Distribution of Cytoplasmic Particulates of Rat Liver

J. F. Thomson, Y. E. Rahman, and F. K. White

Noyes and Smith[1] have presented evidence of a marked effect of ionizing radiation on rat liver mitochondria. Within 15 min after exposure to 1000 r, the numbers of mitochondria per unit weight of liver decreased by about 40% although the total weight of mitochondria per gram dry weight of liver was essentially unchanged. Thus there was an apparent increase of 65% in unit mass per mitochondrion. The effect was transitory, the mitochondrial count returning to normal within 6 hr.

We felt that such a dramatic change should be detectable by gradient centrifugation of liver homogenates. Since a 65% increase in mass would mean an 18% increase in diameter, a difference readily discernible by our procedure,[2] there should be a significant alteration in the distribution pattern of mitochondria-linked enzymes, with an increase in the particle diameter associated with maximum activity.

Methods

Female Sprague-Dawley rats, 8 months old, were used in these experiments. Irradiated rats received 1000 r of X-radiation, delivered at a rate of 200 r/min. The animals were killed 90 min after exposure, the time interval for which Noyes and Smith described the most consistent effects. Both control and irradiated rats were fasted 18 to 20 hr before they were killed.

Most of the methods employed in preparing the tissues, centrifuging, and assaying for enzymatic activity have been described in detail elsewhere.[1] The assay for acid phosphatase was slightly modified from that previously used in that the liberation of bound activity was accomplished by adding a non-ionic detergent, Igepal CO-630, to the reaction mixture, rather than by repeated freezing and thawing.

Results

Distribution of Succinic Dehydrogenase. Figure 48 shows the distribution of succinic dehydrogenase in respect to particle size in control and irradiated animals. Since this enzyme seems to be associated exclusively with mitochondria, Figure 48 thus represents the distribution

pattern of mitochondria. It is clear that there is very little difference between control and irradiated rats. After irradiation, there was a slight decrease in size associated with maximum activity, rather than the increase anticipated from the results of Noyes and Smith. This decrease was from 0.74 to 0.71 μ, and represents a change of less than 10% in mitochondrial volume. The amount of succinic dehydrogenase activity found in the sediment, representing all particles larger than those represented in Figure 48, was about the same in both cases, 31.5% of the total activity in the case of the irradiated rats, 30.3% for the controls. The total activity of the liver homogenate was unchanged by irradiation.

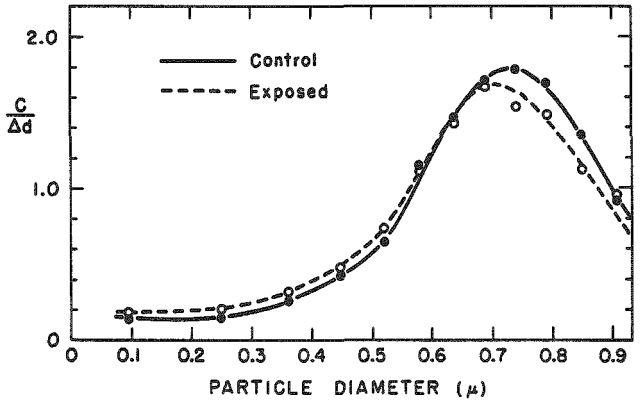

Figure 48. Distribution of succinic dehydrogenase in control and irradiated rat liver. Centrifuged at 3000 rpm for 1 hr; 5 rats per group

Uricase Activity. The two distribution curves for uricase were virtually indistinguishable (Figure 49), and the total activities did not differ significantly. Thus there is no evidence for any alteration of the properties of the particles containing uricase.

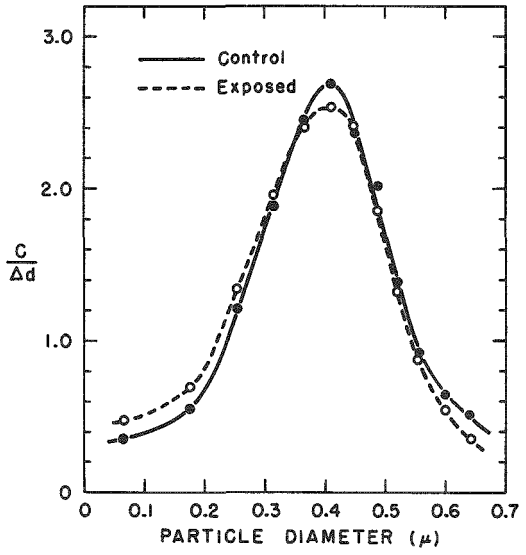

Figure 49

Distribution of uricase in control and irradiated rat liver. Centrifuged at 3000 rpm for 2 hr; 6 irradiated, 4 control rats

Acid Phosphatase Activity. The assay for this enzyme is not so precise and sensitive as that for uricase or succinic dehydrogenase, and considerable variation was encountered among the control and irradiated groups. However, there seemed to be no effect of irradiation on either the activity or distribution of free, bound, or total acid phosphatase. More work needs to be done on the effect of irradiation of lysosomal enzymes, but it may be tentatively suggested that radiation has little effect on liver lysosomes.

Discussion

There are several possible reasons why, in contrast to Noyes and Smith,[1] we have failed to demonstrate a clear-cut effect of radiation on rat liver mitochondria. Noyes and Smith used male Long-Evans rats, fasted 12 hr before exposure, whereas we used female Sprague-Dawley animals, fasted 18 to 20 hr. They exposed their animals to Co^{60} γ-rays at a rate of 35 r/min, while we used 200 r/min of X-radiation, so that our animals were confined to exposure boxes a much shorter time.

What may be of greatest significance, however, is their technique of preparation of subcellular fractions. The method of Smith[3] consists of first forcing the tissue through a disk perforated with 0.9-mm holes, a procedure which we have found to be deleterious to mitochondria.[2] The pulp is then homogenized in 3 volumes of KCl, diluted to about 4 volumes, and centrifuged to sediment the nuclei, mitochondria, cellular debris, and probably a portion of the microsomes into a single pellet. The mitochondria are then extracted from this sediment by 5.75% mannitol (about 0.3 M).

It seems to us that any tendency toward increased fragility of mitochondria would be greatly enhanced by this procedure. In contrast, our technique, employing 0.25 M sucrose and avoiding the use of solutes such as KCl that would cause agglutination, is considerably less traumatic. It was our experience a few years ago that the damage to liver mitochondria produced by feeding carbon tetrachloride was considerably magnified when the liver was mashed before homogenization.

Thus it is possible that there are subtle, transitory changes produced in mitochondrial structure after irradiation, possibly mediated through the endocrines,[4] which are not made evident by our techniques, but which are intensified by the more traumatic procedure of Noyes and Smith.

We are currently studying spontaneous swelling of mitochondria isolated from livers of irradiated and unirradiated rats. As yet, the data are too fragmentary to permit us to draw any conclusions.

References

1. Noyes, P. P., and R. E. Smith. Quantitative changes in rat liver mitochondria following whole body irradiation. Exptl Cell Research 16 15-23 (1959).

2. Thomson, J. F., and F. J. Klipfel. Further studies on cytoplasmic particulates isolated by gradient centrifugation. Arch. Biochem. Biophys. 70 224-238 (1957).

3. Smith, R. E. Quantitative relations between liver mitochondria metabolism and total body weight in mammals. Ann. N. Y. Acad. Sci. 62 403-422 (1956).

4. Benjamin, T. L., and H. T. Yost, Jr. The mechanism of uncoupling of oxidative phosphorylation in rat spleen and liver mitochondria after whole-body irradiation. Radiation Research 12 613-625 (1960).

STUDIES ON THE EFFECTS OF DEUTERIUM OXIDE

IX. The Effects of Various Hormones and Vitamins on the Survival of Mice Maintained at Toxic Levels of D_2O

D. M. Czajka, J. J. Katz* and A. J. Finkel

Abundant evidence exists that mammals can survive deuteration to the extent of 25% in the body fluids, but that replacement of more than one-third of the hydrogen in the body fluids leads to death. The experiments described here were undertaken to ascertain whether the susceptibility of mammals to deuterium could be substantially altered. We have examined the effects of a number of hormones and vitamins for possible effects on the ability of mice to survive toxic concentrations of deuterium in the body fluids. Preliminary results indicate that certain of these substances do in fact substantially enhance the survival of mice under these conditions.

Methods

Fully adult CF No. 1 female mice were used in all the experiments. Four series of mice that were maintained on 75% deuterium oxide drinking water received intraperitoneal or subcutaneous injections of various substances. These injections were begun on the fourth day of deuteration and continued daily, except as noted, for the life of the animal. From other experiments, it was known that the animals would have reached approximately 65% of the normally lethal tissue concentration (over 35%) in four days.(1)

Because the median survival times of control and treated groups were very similar when the animals drank 75% D_2O, the substances that appeared to increase survival were tested again under less stressful conditions, i.e., 50% deuterium oxide drinking water. Because of the lower D_2O concentration in the drinking water, 2 additional days are required to reach 65% of the normally lethal concentration.(1) Consequently, these animals were not injected until the sixth day, and the injections were continued at least until the median survival time was established.

Doses (see Tables 23 and 24) of various substances were calculated from established human doses with the exception of the Aldactone, which was calculated from the dose given to rats.** The water-soluble vitamins were prepared with distilled water so that each mouse received 0.15 ml/day intraperitoneally.

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*Chemistry Division

**Manufacturer's information

TABLE 23

Results of experiments on mice maintained on 75% deuterium oxide drinking water

Group	No. of mice	Dose	Method of administration	Frequency of injection	Median survival time, days
Control	12	-	-	-	9
ACTH	10	0.3 USP units/kg	Intraperitoneal	Daily	10
Cortisone acetate	10	5 mg/kg	Intraperitoneal	Daily	9
Vitamin B mixture*	10	0.15 ml/mouse	Intraperitoneal	Daily	11
Control	12	-	-	-	10
Aldactone**	10	1.65 mg/kg	Subcutaneous	Daily	9
Thyroprotein	10	1.786×10^{-2} mg/kg	Intraperitoneal	Daily	12
Control	10	-	-	-	10
Desoxycorticosterone acetate	10	0.001 mg/mouse	Subcutaneous	Daily	10
TSH†	10	0.1 USP unit/kg	Intraperitoneal	Daily	11
Calcium gluconate	10	2 mg/mouse (4 days) 1 mg/mouse (remainder)	Intraperitoneal	Daily	13
Control	15	-	-	-	13
Parathyroid hormone	15	0.1 units/mouse	Intraperitoneal	Daily	12
Dihydrotachysterol	15	9.37×10^{-4} mg/mouse	Subcutaneous	Every 3rd day	11
Vitamin D_2	15	20 units/mouse	Subcutaneous	Every 3rd day	15

*Each ml contained the following: 2 mg thiamine; 0.066 mg riboflavin; 2 mg nicotinamide; 0.8 mg pantothenic acid; 2μg B_{12}; and crude liver extract.

**Supplied through the courtesy of G. D. Searle and Company.

†Thyroid stimulating hormone.

TABLE 24

Results of experiments on mice maintained on 50% deuterium oxide drinking water

Group	No. of mice	Dose	Method of administration	Frequency of injection	Median survival time, days
Control	20	-	-	-	21
Thyroprotein	15	1.786×10^{-2} mg/kg	Intraperitoneal	Daily	34
Vitamin B mixture	15	0.15 ml/mouse	Intraperitoneal	Daily	61
Control	10	Saline	Intraperitoneal	Daily	14
B_{12}	10	0.3 μg/mouse	Intraperitoneal	Daily	35
Thiamine B1	10	0.3 mg/mouse	Intraperitoneal	Daily	36
Riboflavin B_2	10	0.099 mg/mouse	Intraperitoneal	Daily	37
Pyridoxine B_6	10	0.15 mg/mouse	Intraperitoneal	Daily	42
Vitamin B mixture*	10	0.15 ml/mouse	Intraperitoneal	Daily	44
Control	16	-	-	-	20
Pantothenic acid	18	0.075 mg/mouse	Intraperitoneal	Daily	19
Calcium gluconate	18	1 mg/mouse	Intraperitoneal	Daily	29
Nicotinamide	18	0.75 mg/mouse	Intraperitoneal	Daily	33
Ascorbic acid	18	0.15 mg/mouse	Intraperitoneal	Daily	38

*Each ml contained the following: 2 mg thiamine; 0.066 mg riboflavin; 2 mg nicotinamide; 0.8 mg pantothenic acid; 2 μg B_{12} and crude liver extract.

Vitamin D_2, dihydrotachysterol, and desoxycorticosterone acetate were dissolved in sesame oil and injected subcutaneously in a volume not greater than 0.05 ml/mouse/day. Aldactone was dissolved in Mazola oil and injected in the same volume. Various hormones were injected on a mg/kg or units/kg basis.

Liver samples for deuterium analysis were obtained from recently dead mice whenever possible. Urine samples were obtained from the first group of mice drinking 50% D_2O. The samples were analyzed for deuterium content by methods of infrared spectrophotometry.(2)

Results

Table 23 gives the number of animals, administered dose, method of administration, frequency of injection and median survival times for the animals maintained on 75% D_2O. None of these groups was able to maintain its starting body weight. In spite of supportive therapy, the mice still showed the typical symptoms of high-level deuteration: loss of weight, blepharitis, lack of grooming, convulsions and eventual lenticular cataracts.

When data from the first two groups of mice indicated that some beneficial effects were obtained from thyroprotein and vitamin B mixture, these materials were retested on animals maintained on 50% D_2O drinking water. Here the vitamin B mixture proved to be more effective in prolonging the lives of the animals than did the thyroprotein (Table 24).

Before it became apparent that maintaining animals on 75% D_2O was too stressful, two additional groups were started (Table 23). These trials showed that calcium gluconate, TSH, and Vitamin D_2 have some effect in counteracting the toxic effects produced by D_2O.

Table 24 gives the administered dose, method and frequency of administration and median survival times for the animals maintained on 50% deuterium oxide. Again none of the groups was able to maintain its starting weight. When the treatment appeared to help the animals, they seemed to be calmer and better groomed although many still exhibited convulsive seizures.

The deuterium analysis of urine samples taken from the first group maintained on 50% D_2O showed no differences between the control mice and the experimental animals. All of the surviving animals in this group were sacrificed at 62 days and showed no differences in the amount of distillable deuterium in the various tissues. There was also no difference in the amount of distillable deuterium in the liver samples obtained from recently deceased animals.

With longer survival of the mice as a result of various supportive treatments, there was an increase in the number of lenticular cataracts seen. Many of these animals appeared to be grossly healthy except for complete blindness.

Discussion

It appears from the results shown in Table 23 and particularly in Table 24 that several of the agents tested have a marked effect on the median survival time. These are thyroprotein, vitamin B mixture, vitamin B_{12}, thiamine, riboflavin, and pyridoxine, calcium gluconate, nicotinamide, ascorbic acid and vitamin K. A vitamin B mixture appeared more efficacious than any single known vitamin constituent. The other materials that were tested appeared to have little effect. The substances that have the most pronounced effect are the water-soluble vitamins of the B group and thyroglobulin. The mechanisms by which these substances enable the mice to survive is completely unknown and may, in fact, be related to the ability to survive on a reduced intake of water. It does appear, however, that the animals receiving the effective agents do survive for distinctly longer periods of time at deuterium concentrations that are lethal in controls.

References

1. Finkel, A. J., J. J. Katz and D. M. Czajka. Unpublished data.

2. Katz, J. J., H. L. Crespi, R. J. Hasterlik, J. F. Thomson and A. J. Finkel. Some observations on biological effects of deuterium with special reference to effects on neoplastic processes. J. Natl. Cancer Inst. 18 641-659 (1957)

STUDIES ON THE EFFECTS OF DEUTERIUM OXIDE

X. Clinical Course of Deuterium Intoxication in Dogs

D. M. Czajka, A. J. Finkel, C. S. Fischer,* J. J. Katz**
and B. M. Van Dolah*

All previous work to determine the effects of deuterium oxide on mammals has been done on small animals. The results obtained from such experiments on mice and rats strongly suggested that a number of points could be checked only by working with a larger mammal. We have, therefore, carried out a preliminary investigation of the effects of deuterium on dogs. The beagles used in this work are the largest animal to have been deuterated as far as we know, and a number of observations have now been made that raise new and interesting questions on the physiological effects of deuterium.

Methods

The dogs used in this study were purebred, adult, male beagles. During the control period, the dogs were maintained on drinking water consisting of 50% distilled and 50% tap water. The dogs received dry commercial dog food moistened with beef soup that had been previously diluted with an equal amount of distilled water. The amounts of water and food consumed were measured daily.

Deuteration of the first dog (A19E) was effected by replacing the distilled water with deuterium oxide in both food and drink. After three days the dog refused to eat, became nervous and appeared generally ill. Despite attempts to maintain his intake of the mixture of deuterated food, the dog did not eat well and after 11 days was maintained on regular dog food, receiving deuterium only in his drinking water. After 23 days, the concentration of the deuterium in the drinking water was changed to 60%, and after 43 days the concentration was raised to 75%. After 51 days, the dog was returned to regular water, and the course of recovery was observed.

The second dog (A23A) was also started on 50% deuterium oxide drinking water. After $6\frac{1}{2}$ days, the deuterium concentration was increased to 75%, at which level it remained for a further $5\frac{1}{2}$ days. The dog was maintained on the dry food plus beef soup plus deuterium oxide mixture for the whole period. This dog was found dead on the morning of the 13th day.

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*Health Division

**Chemistry Division

All blood samples were drawn from the external jugular vein with the animals in a fasting state. Blood samples were drawn twice a week while the animals were on deuterium oxide intake; samples were taken from dog A19E only once weekly after the animal was returned to regular water. The following blood tests were run on both animals: hemoglobin, hematocrit, red blood count, white blood and differential count, sedimentation rate, non-protein nitrogen (NPN),[1] serum glucose,[2] blood urea nitrogen (BUN),[3] serum total cholesterol and cholesterol esters,[4] serum glutamic pyruvic transaminase (SGP-T),[5] and deuterium content.[6] In addition, serum sodium,[7] potassium,[7] and chloride[8] were measured on the first dog, and reticulocyte counts and serum glutamic oxalacetic transaminase (SGO-T)[9] concentrations were measured on the second dog.

After a preliminary training period, electrocardiograms were taken with the unanesthetized dog lying on his right side. Standard limb leads, aVR, aVL, aVF, plus four chest leads were taken each time. The chest leads were positioned in accordance with the recommendations of Lombard and Witham,[10] i.e., V_1 and V_2 2 cm from the midline at the level of the 4th intercostal space; V_6 in the same plane at the midaxillary line, and V_4 midway between V_2 and V_6.

An attempt was made to collect 24-hr urine samples on dog A19E, but the results were inconclusive because the animal retained urine until after it was released from the metabolism cage.

Motion pictures of both animals were taken to record the general condition and appearance of the animal and any possible neuromuscular or behavioral changes resulting from deuteration.

Dog A23A was autopsied and tissues were fixed in Zenker-formol solution. Tissue samples were taken for deuterium analysis. The cytological results of the examination of the testis and epididymis from this animal are reported elsewhere.[11]

Results

Dog A19E reached a peak serum deuterium concentration (24.8%) on the seventh day. From day 7 to day 17, the deuterium concentration of the serum dropped to 18.2% and remained within 2% of this level for the remainder of the time that he was maintained on deuterium oxide. The fluid intake of the dog varied from day to day, and the amounts consumed were always less than those during the predeuteration period. For a month after return to regular water, the dog never consumed as much as it had prior to deuteration.

By the 45th day of deuteration, the hemoglobin, hematocrit and red blood count of dog A19E dropped from mean control values of 16.9, 54, and 7.14 million to 10.9, 35.5 and 4.21 million, respectively. Thirty-seven days after return to regular water these values were 16.7, 56, and 5.86 million, and after 91 days, 17.8, 58 and 8.13 million. The white blood count showed two peaks of 21,000 (at days 17 and 28) but otherwise remained within the predeuteration range of 8,600 to 14,000. The differential count showed an increased number of polymorphonuclear leucocytes and a decreased number of lymphocytes as deuteration progressed. The sedimentation rate showed two peak values, 19 and 45, when the white count was above normal. The second peak was reached on day 28, and although the values dropped steadily, they did not return to normal until after the dog had been on regular water 28 days. The serum glucose showed an irregular downward trend with a low of 79 mg %. The NPN, BUN, sodium, potassium and chloride values fluctuated within normal limits during the course of the experiment. The total cholesterol concentration dropped from the control range of 254 to 292 mg/100 ml to 148 on the 36th day of deuteration. The cholesterol esters remained within normal limits, with the exception of the value obtained on the 38th day, at which time it was more than twice the control value. Further details are given in Table 25.

TABLE 25

Clinical laboratory data on dog A19E

		Sed. rate, mm/hr	RBC millions	WBC	Hgb g%	Hemat., %	NPN, mg%	BUN mg%	Serum glucose, mg%	Serum Na, meq/liter	Serum K, meq/liter	Serum Cl, meq/liter	Serum cholesterol mg 100 ml	
													Total	Esters
Pre-deuteration control range		9.5 1.0	6.75- 7.73	8,600- 14,000	15.2- 17.6	50- 58	25.8- 34.5	11.6- 33.0	95- 106	136- 145	4.2- 4.6	115- 122	254- 292	36- 61
Days maintained on D_2O ingestion	1	1	7.12	11,900	16.1	51	26.7	18.6	101	145	4.3	115	250	47
	3	1	7.15	8,300	16.2	52	30.6	19.5	106	152	4.0	112	280	46
	7	0	6.57	8,200	16.3	53	38.0	19.8	90.5	143	4.2	104	282	50
	10	0	6.36	8,600	16.5	51	26.9	16.0	87	135	4.9	111	235	29
	13	0	5.70	8,300	13.9	49	32.1	18.4	87	149	4.3	104	223	36
	17	19	5.64	20,700	13.3	44	31.6	14.0	85	144	3.7	107	290	39
	24	10	5.23	12,300	12.8	40	30.5	21.0	111	148	3.7	120	194	40
	28	45	4.88	21,000	11.1	38	40.7	17.2	94.5	147	3.9	97	218	35
	31	35	3.48	18,500	11.3	37	35.7	18.8	79	161	4.0	97	200	28
	36	20	4.57	12,450	11.3	38	26.0	20.0	109	149	4.8	110	148	22
	38	23	4.26	13 600	11.6	38	27.9	18.1	91	147	3.9	110	190	120
	43	12	4.53	10,900	11.4	37	29.6	16.1	94	147	4.4	104	186	64
	45	12	4.21	12,700	10.9	35.5	32.5	19.5	86	146	4.2	107	162	30
	49	5	4.39	13 300	11 5	38	31 2	21.0	93	147	4.4	106	160	24
Days after return to ordinary water	2	13	4.41	12,600	11.7	37	27.0	18.1	95	-	-	-	151	30
	7	8	5.26	12,800	11.8	37	20.9	17.3	96	144	4.6	106	180	27
	13	3	4.90	11,600	13.5	42	24.6	18.5	104	-	-	-	201	37
	20	3	5.35	9 600	15.3	49	23.3	16.0	115	142	4.5	110	198	36
	28	0	5.83	11,200	15.3	52	25.8	21.5	86	-	-	-	202	31
	37	0	5.86	8,500	16.7	56	20.6	17.6	104	145	4.4	112	240	33
	91	1.5	8.13	10.700	17.8	58	-	20.5	107	-	-	-	250	78

Due to the sudden death of the second dog (A23A) only a few clinical data are available (Table 26). The hemoglobin, red blood count, SGP-T, SGO-T, and cholesterol remained within normal limits during the 12 days of deuteration. The hematocrit, NPN and BUN were elevated. The WBC was below normal on day 4, but returned to normal by day 10. Again the differential count showed an increased number of polymorphonuclear leucocytes and a decreased number of lymphocytes. The glucose concentration dropped rapidly and reached a minimum value of 66.5 mg % on the 10th day. The deuterium content of the serum was 20% on day 4, 25.6% on day 7, 35.5% on day 10 and 33% post mortem. The dog did not show any gross pathological changes at autopsy. Microscopic examination of the tissues has not been completed at this time.

TABLE 26

Clinical laboratory data on dog A23A

	Control range	Days on D_2O		
		4	7	10
D_2O in serum, %	-	20.0	25.6	35.2
Hemoglobin	12-14	14.2	14.5	14.7
Hematocrit	42-46	48	52	54
RBC, millions	5.18-6.48	5.81	5.42	5.59
WBC	10,200-11,500	6,300	7,100	11,200
Differential				
Polys.	52-78	61	70	83
Lymphs.	13-34	26	20	9
Eosin.	2-9	7	6	1
Monos.	2-5	4	2	3
Stabs.	2-4	2	2	4
Sed. rate, mm/hr	3-18	0.5	0	0
Reticulocyte count	0.5-1.1	0.7	-	0.2
BUN, mg %	13-18	12.5	13.3	18.5
NPN, mg %	22-31	21.3	27.9	33.5
Serum glucose, mg %	93-124	89	71	66.5
SGO-T*	32-132	16	-	24
SGP-T**	34-122	29	-	37
Cholesterol, total	300-366	280	322	282

*Arbitrary units as defined in Reference 9

**Arbitrary units as defined in Reference 5

The electrocardiograms of both animals showed myocardial changes that were similar to those produced by hypothyroidism or hypocalcemia. After seven days on deuterium, dog A19E showed inverted T waves in leads 1 and aVL. The same tracing also revealed peaked T waves in leads 2, 3, V_4, V_6 and aVF. After 10 days, the P wave was diphasic and inverted in lead 3; T was now coved upward in lead 1, and the ST segment was elevated in V_1, V_2, V_4 and V_6. On the 24th day, the changes were not as pronounced; T wave inversion in leads 2, 3, and aVF was not as deep and the secondary T in V_1 was now upright. By the 45th day, the tracing was similar to predeuteration electrocardiograms. Thus the myocardial changes appear to be fully reversible.

Similar changes were noted with dog A23A, and both dogs showed a moderate bradycardia relative to the control predeuteration tracings. The QT interval increased somewhat in the first dog, with a maximum at the 14th day, while in dog A23A, it increased fairly steadily with duration of deuteration to a maximum in the last tracing on the 12th day.

The motion pictures of the dogs revealed muscular weakness which was particularly apparent in the hind legs of dog A19E. Usually the dog was very animated and would jump against the enclosure; however, after deuteration, the dog would drop down as if unable to bear the weight on the hind limbs. Only after much urging could the animal be induced to walk around the enclosure. Muscular tremors were not observed in the first dog, but were very obvious in the second during the two days prior to death. Although dog A23A died unobserved, the condition of the animal suggested the possibility that death occurred during a convulsive seizure.

Discussion and Summary

Although the two dogs were maintained in a highly deuterated state under different conditions, similar changes were produced in each. The most notable of these findings were unaltered white blood cell counts with relative lymphopenia and increase in polymorphonuclear leucocytes, hypoglycemia related to the degree of deuterium level in the body, electrocardiographic changes indicative of myocardial abnormality, and generalized muscular weakness. The second dog died too quickly for other changes to manifest themselves. In the first dog, the changes included the delayed drop in total serum cholesterol and the elevation in sedimentation rate. On the other hand, the elevated NPN, BUN and hematocrit seen in the second dog may have been related to the hemoconcentration resulting from rapid and toxic deuteration. Further studies are contemplated to clarify the nature of the clinical changes at sublethal levels of deuterium intoxication in dogs. The nature of the results reported here, which in the aggregate could only have been seen on a large animal, strongly confirms the desirability of large animal studies in this field.

References

1. Rappaport, F. Rapid Microchemical Methods for Blood and CSF Examinations. New York: Grune and Stratton, Inc., 1949. pp. 149-151.

2. Fister, H. J. Method D 12A, in Manual of Standardized Procedures for Spectrophotometric Chemistry. New York: Standard Scientific Supply Corporation, 1950.

3. Fister, H. J. Method B-25, in Manual of Standardized Procedure for Spectrophotometric Chemistry. New York: Standard Scientific Supply Corporation, 1950.

4. Fister, H. J. Methods C30 and C31, in Manual of Standardized Procedure for Spectrophotometric Chemistry. New York: Standard Scientific Supply Corporation, 1950.

5. Wroblewski, F. and J. S. LaDue. SGP-T in hepatic and cardiac disease: a preliminary report. Proc. Soc. Exptl. Biol. Med. 91 569-71 (1956).

6. Katz, J. J., H. L. Crespi, R. J. Hasterlik, J. F. Thomson and A. J. Finkel. Some observations on biological effects of deuterium with special reference to effects on neoplastic processes. J. Natl. Cancer Inst. 18 641-659 (1957).

7. New flame photometric method for sodium and potassium in serum. Beckman Bulletin DU 12B.

8. Knights, E. M., R. P. MacDonald, and J. Ploompu. Ultramicro methods for clinical laboratories. New York: Grune and Stratton, Inc. 1957. pp. 34-39.

9. Steinberg, D., D. Baldwin and B. Ostrow. A clinical method for the assay of serum glutamic-oxalacetic transaminase. J. Lab. Clin. Med. 48 144-151 (1956).

10. Lombard, E. A. and C. Witham. Electrocardiogram of the anesthetized dog. Am. J. Physiol. 181 567-574 (1955).

11. Amarose, A. P., D. M. Czajka, B. R. Nebel and T. S. McConnell. Cytological observations of the male reproductive organs of the mouse and dog following administration of deuterium oxide. Biological and Medical Research Division Semiannual Report, Argonne National Laboratory. ANL-6264 (1959). pp. 130-137.

STUDIES ON THE EFFECTS OF DEUTERIUM OXIDE

XI. Effects of Antitumor Drugs on Ascites Tumors in Deuterated Mice

A. J. Finkel, D. M. Czajka, and J. J. Katz*

In 1933 Fischer[1] reported that mouse carcinoma cells were unable to grow when the deuterium concentration was above 50%. Barbour and Allen[2] observed that the growth of transplanted lymphosarcoma and mammary carcinoma was slower in mice drinking 40% deuterium oxide than in mice drinking ordinary water. Recently, we reported that the growth of Krebs-2A ascites tumor was depressed by the presence of deuterium oxide in the body fluids in concentrations ranging from 13 to 32%.[3] However, the most effective carcinolytic level was also lethal to the host animal. These results led to an exploration of the effects of therapy with known anti-tumor drugs combined with lower, nontoxic levels of deuterium oxide.

Materials and Methods

CF No. 1 young adult female mice were used in all of these experiments. They were maintained on 30% D_2O drinking water for 7 days prior to inoculation with 10^7 Krebs-2A ascites tumor cells. All solutions of chemotherapeutic agents were prepared in distilled water in concentrations such that the animals received 0.01 ml/g body weight by intraperitoneal injection. Measurements of tumor growth were made either according to the quantitative methods developed by Patt et al.,[4] or by following weight gain and survival.

Results and Discussion

In the first experiment with 5-fluorouracil, 6 groups of 15 animals each were used. Three of the groups were kept on 30% D_2O in the drinking water for 7 days prior to inoculation with ascites tumor cells, and 3 groups drank tap water. Intraperitoneal injections of either 6.25 or 12 mg/kg 5-fluorouracil were given on the second, third, fourth, and fifth days after tumor inoculation. Control groups, deuterated and nondeuterated, were not injected after inoculation. Five animals from each group were sacrificed on the third, sixth and ninth days after tumor inoculation. Consequently, animals were sacrificed 1 day after a single injection, and 1 and 4 days after 4 injections.

*Chemistry Division

Table 27 gives the results of this experiment. Both levels of 5-fluorouracil were effective in lowering the growth rate of the ascites tumor in animals maintained on ordinary water. There were some differences in the total tumor cell populations of the deuterated and nondeuterated mice receiving the fluorouracil injections but the differences were small and not entirely consistent. The deuterium oxide alone depressed the tumor growth rate when compared to untreated control tumors, a finding noted previously.(3)

Because of these results, in the next experiments we decided to allow an additional day for the tumor to become well established before the series of 4 daily injections were begun, and lower dose rates of fluorouracil were used. The animals were again sacrificed 1 day after a single injection, and 1 and 4 days after 4 injections, i.e., on the fourth, seventh, and tenth days after inoculation. Table 28 gives the combined results of two sets of experiments. It was found that injections of 6 mg/kg of 5-fluorouracil were effective in lowering the growth rate whether or not the animals had been drinking deuterium oxide. Moreover, at this dose level, the mean tumor cell population in the deuterated mice averaged roughly 50% of that of the nondeuterated mice at the fourth and seventh days after inoculation. However, only one animal survived in each group to the tenth day.

Table 27

Effect of 5-fluorouracil and deuterium oxide on Krebs-2A ascites tumor growth in mice

	Fluorouracil mg/kg	Mean total cell population (1×10^6)		
		Day 3	Day 6	Day 9
H_2O	0	92.49	604.30	626.92
	6.25	54.97	213.45	115.43
	12.5	41.62	72.14	57.07
D_2O	0	94.63	335.16	221.05
	6.25	35.12	94.05	142.99
	12.5	42.31	63.83	55.79

TABLE 28

Effect of 5-fluorouracil and deuterium oxide on
Krebs-2A ascites tumor growth in mice

Drinking water	5-fluorouracil, mg/kg	Day 4			Day 7			Day 10		
		No. of mice	Mean AFV* (ml)	Mean TCP** (1×10^6)	No. of mice	Mean AFV* (ml)	Mean TCP** (1×10^6)	No. of mice	Mean AFV* (ml)	Mean TCP** (1×10^6)
H_2O	0	10	1.88	262.64	10	3.94	920.61	8	13.04	1100.70
	2	5	1.43	263.16	5	4.49	596.98	5	12.06	962.37
	3	5	1.48	157.50	5	5.12	317.48	0	-	-
	6	5	2.43	203.85	5	2.82	77.70	5	3.42	1.64
30% D_2O	0	10	1.77	195.64	10	3.02	307.38	6	8.94	565.35
	2	5	1.04	148.16	5	2.57	294.73	5	7.32	441.98
	3	5	1.90	127.93	5	3.23	49.41	1	0.63	0.22
	6	5	1.68	113.45	5	2.32	37.60	1	0.64	0.22

*AFV = ascitic fluid volume (cell-free)

**TCP = total cell population

At the next lower level, 3 mg/kg, there was a greater differential effect on tumor growth when fluorouracil was used in conjunction with deuterium oxide, but most of the surviving mice died between the seventh and tenth days. At still lower levels, 2 mg/kg, fluorouracil alone was only slightly effective in suppressing tumor growth when compared with that in untreated mice, but the presence of deuterium in the body fluids enhanced this effect so that an appreciable depression of tumor growth occurred along with fairly good survival of the host animals.

In order to study the survival patterns in these mice, an additional series of four groups of ten mice each was followed along with the sacrifice series that received 2 mg/kg fluorouracil on 4 successive days. This dose was injected into tumor-bearing mice that were maintained on ordinary water alone or on 30% deuterium oxide. Appropriate untreated control groups were also followed. Weight gain and median survival time are plotted in Figure 50. The arrows indicate the median survival time for each group. Survival was poorest (14-day median) among mice maintained on ordinary drinking water, with or without fluorouracil injections. Survival was greatest (28-day median) among animals drinking 30% heavy water, and was somewhat less among deuterated mice injected with fluorouracil (24-day median). Peak weight gain occurred at the 18th to 20th day after inoculation and was greatest in mice receiving D_2O alone. Weight gain was least in mice receiving low level injections of fluorouracil alone.

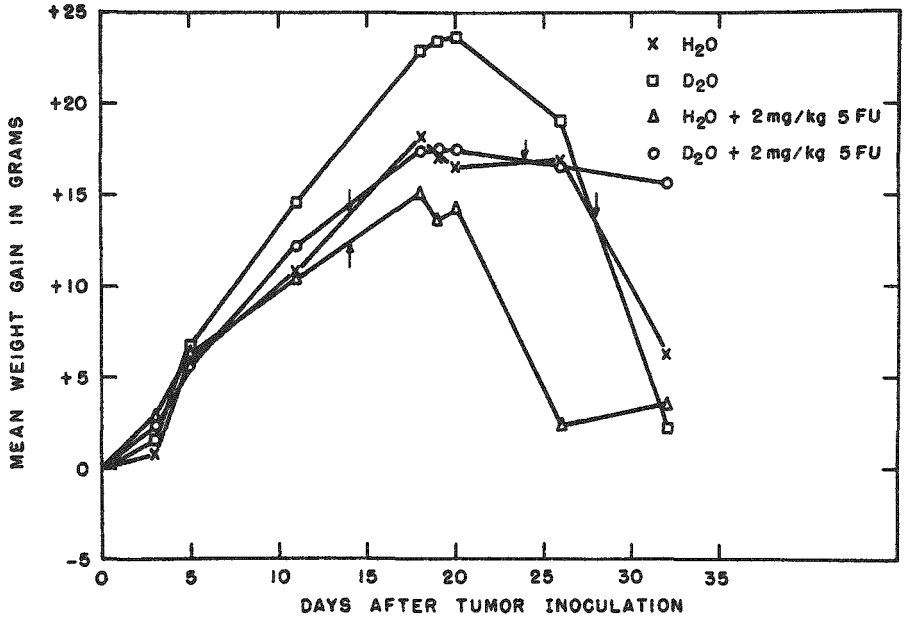

Figure 50. Mean weight gain and median survival time (shown by arrows) for mice inoculated with Krebs-2A ascites tumor; 5-fluorouracil, 2 mg/kg injected intraperitoneally on third, fourth, fifth and sixth days after tumor inoculation.

In the foregoing experiments, the 5-fluorouracil was injected for four successive days shortly after tumor inoculation. In order to assess the effects of prolonged treatment with injections of small amounts of this compound, an additional experiment was designed in which 2 mg/kg was given intraperitoneally daily for 10 days starting with the third day after tumor inoculation. Figure 51 illustrates the weight gain and survival data from this experiment. Median survival time was greatest (23 days) with 30% D_2O alone, and slightly less (19 days) among the untreated mice drinking ordinary water. Fluorouracil-treated animals, whether deuterated or not, had identical median survival times (13 days), but the non-tumor-bearing mice were unaffected by this level of chemotherapy, with or without deuterium. In the tumor-inoculated mice, repeated fluorouracil injections markedly suppressed weight gain and, by inference, tumor growth. Up to the time when 50% of the mice were dead, this effect appeared to be slightly greater in deuterated animals; it cannot be considered dependable beyond this point.

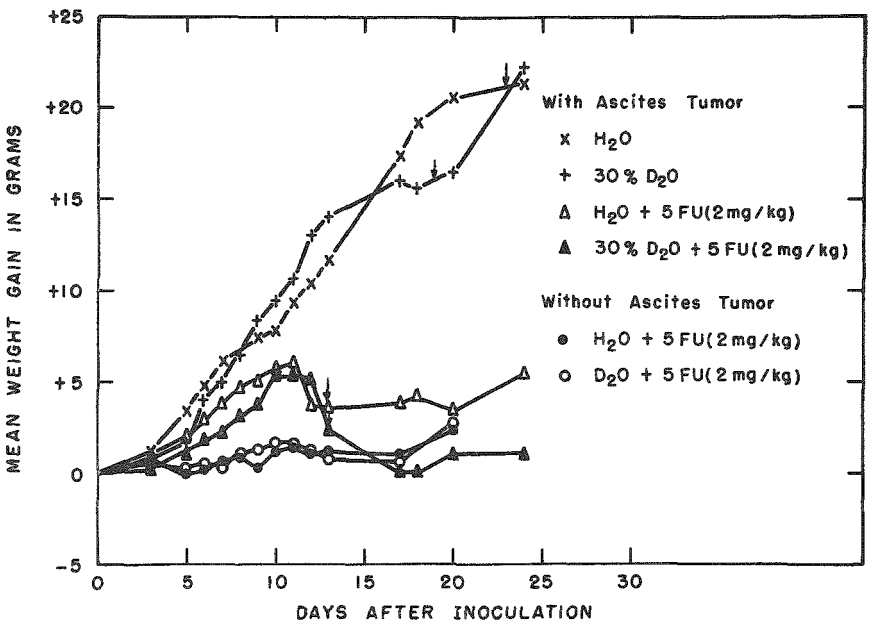

Figure 51. Mean weight gain and median survival time (shown by arrows) for mice inoculated with Krebs-2A ascites tumor and for non-tumor control mice; 5-fluorouracil, 2 mg/kg, injected intraperitoneally daily for 10 days starting with 3rd day after tumor inoculation.

It appears that otherwise nontoxic levels of fluorouracil can have profound effects in suppressing Krebs-2A ascites tumor growth in mice. While this effect may be enhanced by prior or concomitant deuteration of the organism, the combined action leads to the death of the host earlier than it would otherwise occur. It is possible that this result may be the consequence of accumulation of breakdown products from tumor cells that have been damaged by the deuterium and fluorouracil. On the other hand, tumor growth may lag in these animals because general metabolic activities

may be slowed as a result of partial replacement of hydrogen by deuterium in the body fluids, so that neither the host nor the tumor can be maintained. In this case, tumor growth is controlled but the host succumbs.

Similar results were obtained with 6-mercaptopurine, which was injected intraperitoneally into mice at a dose level of 5 mg/kg on the third, fourth, fifth, and sixth days after tumor inoculation. The weight gain data are illustrated in Figure 52. Weight gain was not affected by deuteration alone, nor by this level of mercaptopurine alone, but was markedly suppressed by the administration of mercaptopurine to deuterated tumor-bearing mice. However, in the latter case, median survival time was 12 days, while in the other groups it was beyond 26 days.

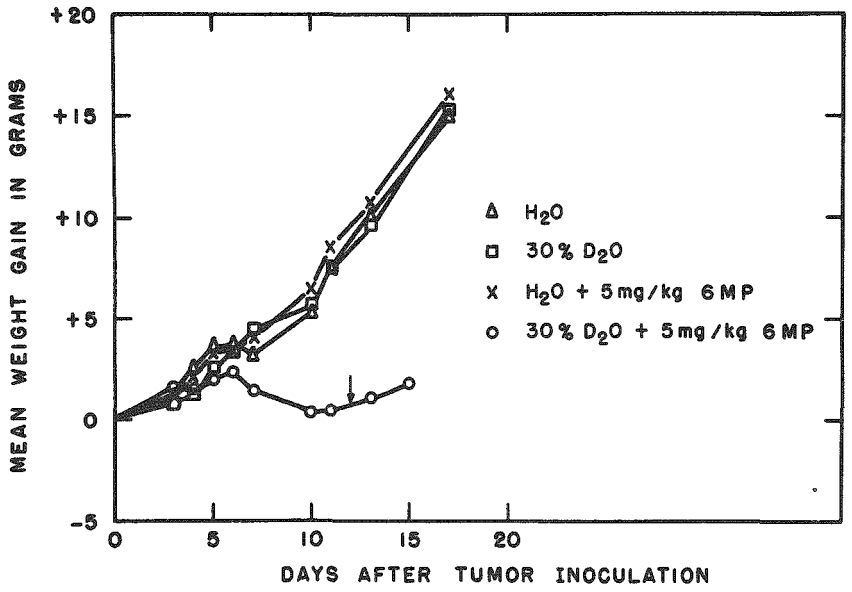

Figure 52. Mean weight gain and median survival time (shown by arrow) for mice inoculated with Krebs-2A ascites tumor; 6-mercaptopurine, 5 mg/kg injected intraperitoneally on third, fourth, fifth and sixth days after tumor inoculation.

These experiments with 5-fluorouracil and with 6-mercaptopurine indicate that an enhancement of antitumor activity can be accomplished when partial deuteration of the organism accompanies the administration of the drug. Deuteration in these experiments has been at a level which per se is nontoxic for mice,[5] and the drugs have been studied at dose levels that are largely ineffective in themselves. It would appear that deuterium, through the action of the mass isotope effect, somehow affects metabolism so that the effects of antitumor compounds are altered. These preliminary results with combined therapy appear to be sufficiently promising to warrant further attention.

Miss Betty Van Dolah, Health Division, provided valuable technical assistance in connection with tumor cell counts.

References

1. Fischer, A. Wachstum von Gewebezellen in schwerem Wasser. Protoplasma 26 51-55 (1936).

2. Barbour, H. G. and E. Allen. Tumor growth in mice one-fifth saturated with deuterium oxide (heavy water) Am. J. Cancer 32 440-446 (1938).

3. Finkel, A. J. and D. M. Czajka. The effect of deuterium oxide on ascites tumor growth in mice. Ann. N. Y. Acad. Sci. 84 755-762 (1960).

4. Patt, H. M., M. E. Blackford and J. L. Drallmeier. Growth characteristics of the Krebs ascites tumor. Proc. Soc. Exptl. Biol. Med. 83 520-524 (1953).

5. Finkel, A. J. and D. M. Czajka. Studies on effects of deuterium oxide. V. Growth data for mice. Biological and Medical Research Division, Semiannual Report, Argonne National Laboratory. ANL-5841 (1957), p. 160.

EFFECTS OF DEUTERIUM ON SOME ENZYMATIC REACTIONS

J. F. Thomson, D. A. Bray* and F. K. White

Although it is highly improbable that the toxic action of deuterium in mammals can be ascribed to an interference with any one mechanism, it is nevertheless of interest to survey a number of representative biochemical reactions in vivo and in vitro. We have previously reported a number of studies on the effect of D_2O and other deuterium compounds on enzymes.[1-5] This communication represents a continuation of these experiments.

Methods

Rat liver was used as the source of catalase, uricase, and tryptophan pyrrolase. A malic dehydrogenase preparation was given to us by Dr. Robert W. Swick, who obtained it from an extract of Propionibacterium shermanii by ammonium sulfate and acid treatment. The reaction was followed by measuring the disappearance of DPNH (or DPND) during the reduction of oxalacetate to malate. DPND was prepared by reduction of DPN by hydrosulfite in D_2O, and was isolated as the barium salt. The DPNH used in these experiments was similarly prepared in H_2O.

Results

Catalase. Increasing the concentration of D_2O in the incubation mixture caused a virtually linear decrease in the rate of decomposition of perborate. In 95% D_2O, the activity was 73% of that observed under control conditions. This decrease is slightly less than that reported by Shibata and Watanabe,[6] whose data indicate that the initial rate of decomposition of hydrogen peroxide was decreased to 64% of the control value in 99% D_2O. In 30% D_2O, the maximum concentration obtainable in vivo for mammals, there was less than 10% inhibition.

Uricase. Three different concentrations of D_2O were tested in these experiments. No effect was observed with 30% D_2O; there was 5% inhibition in 60% D_2O, and 20% inhibition in 94% D_2O. In these experiments, the assays were carried out at several pH values, and the activities at optimum pH (or pD) were used for comparison. Maximum activity in H_2O was observed at pH 9.4; in 95% D_2O, the buffer in which maximum activity was observed gave a reading of pH 9.7.

Tryptophan pyrrolase. D_2O had very little effect on tryptophan pyrrolase. In one experiment, a 25% inhibition was observed; however, we were unable to confirm this observation in three subsequent assays, finding only an 8 to 10% inhibition in 98% D_2O.

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Malic Dehydrogenase. In the case of this enzyme we were able to study both solvent effects and substrate (coenzyme) effects of deuteration. Under conditions of maximum activity, the substitution of DPND for DPNH or of D_2O for H_2O inhibited the reaction about 30%; substitution of both DPND and D_2O caused over 50% inhibition.

We have also studied the reaction at different concentrations of coenzyme, keeping the oxalacetate concentration at saturation level. A Lineweaver-Burk plot of these data is shown in Figure 53. Table 29 shows the Michaelis constants (K) and the maximum velocities (V_m) of the reactions with DPNH and DPND in H_2O and D_2O. The use of D_2O as a solvent caused an increase in the Michaelis constants and a decrease in the maximum velocities for both DPNH and DPND. On the other hand, the substitution of DPND for DPNH decreased the values of both K and V_m in both solvents.

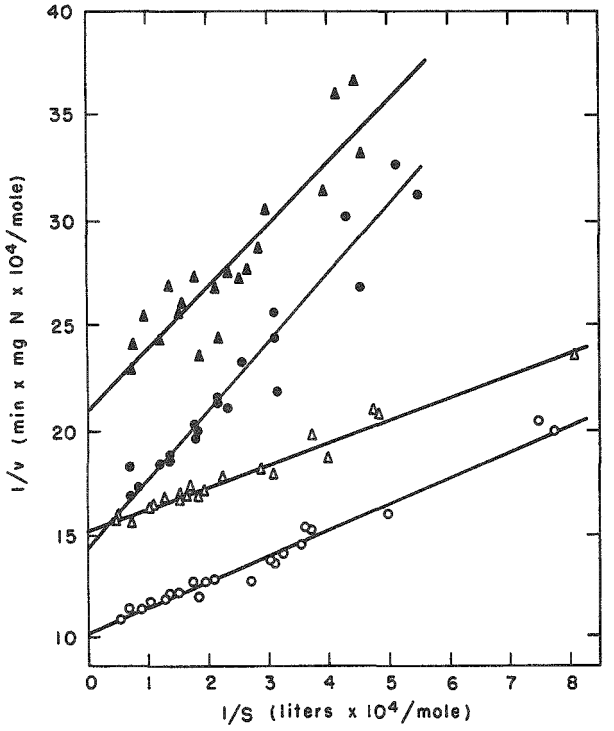

Figure 53. Estimation of Michaelis constant and maximum velocity for malic dehydrogenase-DPNH, with DPNH and DPND as coenzymes, in H_2O and in D_2O. S is the concentration of coenzyme and v is the reaction velocity.

TABLE 29

Michaelis constants and maximum velocities for malic dehydrogenase

Coenzyme	Solvent	K (1×10^{-5} M)	V_m (μM/min/mg N)
DPNH	H_2O	1.22	9.78
DPND	D_2O	1.42	4.78
DPNH	D_2O	2.29	6.95
DPND	H_2O	0.71	6.70

Discussion

In the case of catalase it is impossible to separate the solvent effects of deuterium from the substrate effects since deuterium readily exchanges with hydrogen peroxide liberated from sodium perborate, the substrate used in this assay. For uricase and tryptophan pyrrolase, however, there is relatively little effect of D_2O as a solvent, even at very high concentrations. It can thus be presumed that any steps in these reactions involving water are not rate-limiting.

With regard to the studies on malic dehydrogenase, it should be pointed out that the DPND used in these experiments was not stereospecifically labeled, and actually represented a mixture of the α- and β-DPND stereoisomers. Thus the isotope effects described here represent a mixture of primary effects (rupture of C-D bond) and secondary effects (rupture of a C-H bond in a molecule containing deuterium in a nonreacting position). We intend to prepare both α- and β-DPND, and shall repeat these experiments with the individual stereoisomers. It should also be possible to prepare 4,4-d_2-DPNH ($DPND_2$), with both hydrogens in the 4-position of the reduced pyridine ring replaced by deuterium, so that both primary and secondary effects will occur in each molecule of coenzyme participating in the reaction.

References

1. Katz, J. J., H. L. Crespi, R. J. Hasterlik, J. F. Thomson, and A. J. Finkel. Some observations on biological effects of deuterium, with special reference to effects on neoplastic processes. J. Natl. Cancer Inst. 18 641-659 (1957).

2. Thomson, J. F., and F. J. Klipfel. Studies on the enzymic dehydrogenation of deuterated succinate. Biochem. Biophys. Acta 44 72-77 (1960).

3. Thomson, J. F., and F. J. Klipfel. Some effects of D_2O in vivo and in vitro on certain enzymes of rat tissues. Biochem. Pharmacol. 3 283-288 (1960).

4. Thomson, J. F. Fumarase activity in D_2O. Arch. Biochem. Biophys. 90 1-6 (1960).

5. Thomson, J. F. Physiological effects of D_2O in mammals. Ann. N. Y. Acad. Sci. 84 736-744 (1960)

6. Shibata, K., and A. Watanabe. Über die oxydoreduktiven Enzymwirkungen in schwerem Wasser. Acta Phytochimica 15 169-82 (1949).

EFFECT OF D_2O ON PLASMA VISCOSITY AND ERYTHROCYTE FRAGILITY

J. F. Thomson, F. K. White, and D. A. Bray*

Feeding D_2O to rats causes a depression of renal function, both the glomerular filtration rate and the renal plasma flow decreasing to 40% of the control values.[1] Since these changes occur without significant morphologic or metabolic alteration, and since they are readily reversible upon restoration of H_2O to the animal, the effect of D_2O was considered to be indirect, possibly reflecting changes in adrenal activity. Another possible explanation, however, would be an increased viscosity of the blood as a result of replacement of H_2O by the more viscous D_2O. To test this possibility we have studied the viscosity of plasma obtained from rats drinking D_2O. In this same experiment we have also measured the osmotic fragility of erythrocytes obtained from the same animals.

Methods

Female Sprague-Dawley rats, four months old, were given D_2O ad libitum as a 50% solution in tap water. The rats were weighed twice a week, and samples of urine were collected once a week for D_2O analyses.**

Plasma viscosity was measured with Ostwald viscosimeters of 1-ml capacity. All measurements were made at 25.0°C. Plasma protein was determined by nesslerization of an H_2SO_4-H_2O_2 digest of a 10- or 20-μl sample.

Osmotic fragility was measured by adding 0.05-ml aliquots of whole blood or washed cells resuspended in isotonic saline to a series of tubes containing 6 ml of varying concentrations of sodium chloride (0.22 to 0.70%). After standing for 1 hr at 25°C, the tubes were centrifuged, and the degree of hemolysis was determined by colorimetric estimation of hemoglobin in the supernatant. In each experiment the concentration of sodium chloride at which 50% hemolysis occurred was determined; this value we call the HC_{50}. Fragility studies on red cells of deuterated rats were carried out in 30% D_2O.

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**Analyses for deuterium were carried out by Dr. Henry L. Crespi and his assistants, Chemistry Division.

Results

Mortality. The first death occurred on the 18th day of deuteration, and during the next 9 days 6 more rats died, all of the rats dying with 26 to 34% of their body fluids replaced by D_2O. The eighth rat was returned to ordinary drinking water after 28 days, and apparently has made a complete recovery, even though its body weight had dropped from 240 to 188 g. The average weight loss of the deuterated rats was 26 g, whereas the controls gained 12 g during the period of the experiment.

Plasma Viscosity. There was a progressive decrease in plasma viscosity during the course of deuteration; this decrease was associated with loss of plasma protein (Table 30). Figure 54 shows the relationship between viscosity and protein concentration of the plasma samples examined in this experiment. The solid line indicates values obtained by preparing various dilutions of a sample of normal rat plasma with isotonic saline. It is clear that the substitution of some of the H_2O by D_2O had a negligible effect on the viscosity.

Osmotic Fragility. There was little effect of D_2O either *in vivo* or *in vitro* on the hemolysis of rat erythrocytes. For 8 blood samples taken between the 24th and 28th days of deuteration (average urinary D_2O concentration of 30.8%), the HC_{50} was 0.463 $\pm$ 0.012%, in contrast to the control average (11 rats) of 0.457 $\pm$ 0.005%. The effect of 99% D_2O *in vitro* was equally negligible, the HC_{50}'s differing from the values in H_2O by only 0.004% NaCl.

TABLE 30

Effect of D_2O on viscosity and protein concentration of rat plasma

Days on D_2O	Urinary D_2O, %	No. rats	Relative viscosity*	Plasma protein*
0	-	8	1.71 $\pm$ 0.02	7.33 $\pm$ 0.22
14-17	24.1	8**	1.60 $\pm$ 0.01	6.88 $\pm$ 0.45
24	30.8	4**	1.56 $\pm$ 0.01	6.21 $\pm$ 0.03
27	>31	1	1.53	5.50

*Averages and standard deviations

**Viscosities were measured on pooled pairs of samples; thus the data for 14-17 days represent four measurements, those for 24 days two measurements.

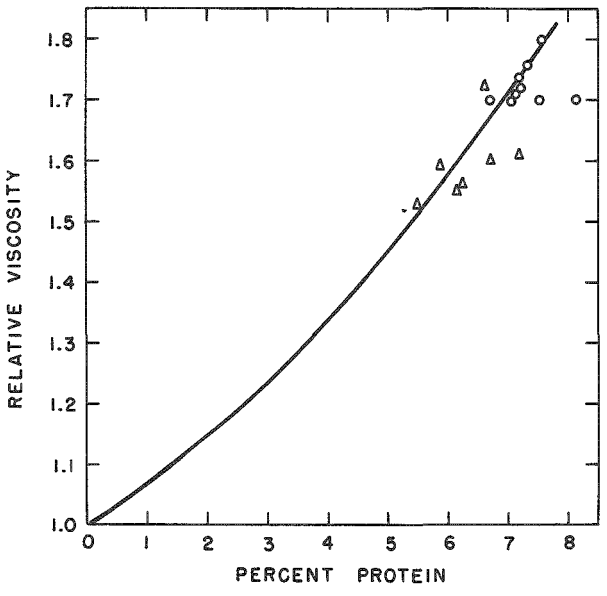

Figure 54. Correlation of viscosity and protein concentration of rat plasma. Circles, control rats; triangles, rats fed D_2O.

Discussion

It is clear that the changes in kidney function observed in deuterated rats cannot be attributable to an increased viscosity of the blood; any increase caused by D_2O itself is more than offset by the marked decrease resulting from loss of plasma protein.

Perhaps it is not surprising that there was no effect of deuteration on erythrocyte fragility. Since there is apparently a complete cessation of erythrocyte production in rats drinking 50% D_2O for more than 10 days,[2] there is little possibility of incorporation of deuterium into non-exchangeable positions in the erythrocyte. The studies with D_2O in vitro showed no effect of exchangeable deuterium on fragility, so that only the influence of aging could possibly be important in these studies.

References

1. Thomson, J. F., and F. J. Klipfel. Changes in renal function in rats drinking heavy water. Proc. Soc. Exptl. Biol. Med. 97 758-759 (1958).

2. Thomson, J. F. Physiological effects of D_2O in mammals. Ann. N. Y. Acad. Sci. 84 736-744 (1960).

ENERGY TRANSFER IN PHOTODYNAMIC ACTION*

D. E. Smith, Leonida Santamaria**
and Bernard Smaller†

Although considerable information is available on the gross aspects of the photodynamic phenomenon,[1] few data relate to the details of the events starting with the absorption of radiant energy by the sensitizer and ending with the oxidation of the biological substrate. Prominent among the suggestions for the formation of intermediates to account for this transfer of energy have been the excitation of the sensitizer to the triplet state and the reaction of the excited sensitizer with oxygen to form a peroxide or a biradical.[1,2] Actual data have been obtained in phosphorescence,[3] paramagnetic susceptibility[4] and flash photolysis studies,[5] however, and these show that triplet states are produced when various sensitizers are irradiated with visible light.

The present study is concerned with the application of electron spin resonance spectroscopy to a model system of photodynamic action in an attempt to determine whether free radicals might constitute some of the intermediates in the transfer of energy in photosensitized oxidation.

Methods

The model system used consisted of hematoporphyrin (sensitizer) (1.2×10^{-3} M) and human blood serum (substrate) (1:3) in phosphate buffer at pH 7.3. The solutions to be irradiated and measured in the microwave spectrometer were contained in Pyrex tubes of 3 mm inside diameter. A high-pressure mercury arc was used as a light source, the light passing through four 1-mm layers of Pyrex glass before reaching the sample. Irradiations were carried out with the solutions held at -20° C or at -196° C. In all instances the samples were quickly transferred at the end of the irradiation to a Dewar flask containing liquid nitrogen.

Electron spin resonance studies were carried out using a 9,000 Mc electron paramagnetic resonance instrument equipped with a lock-in phase-sensitive detection system such that the second derivative of the microwave absorption was recorded. Measurements were made with the samples at the temperature of liquid nitrogen.

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*Presented at the Symposium on Free Radicals in Biological Systems, March 21-23, 1960.

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In some experiments the reducing agents cysteine or ascorbic acid were added to the system in concentrations of 1.2×10^{-2} M prior to irradiation. In other instances the gas content of the samples was altered prior to irradiation by degassing to high vacuum and regassing with helium or nitric oxide.

Results

The results of the experiments in which the irradiations were carried out at -20°C are summarized in Table 31. In no case were electron spin resonance signals observed in the absence of irradiation. After irradiation, signals were obtained only from solutions containing hematoporphyrin. Signals were absent in solutions of hematoporphyrin to which either cysteine or ascorbic acid was added, and they were reduced by two-thirds in samples to which serum was added prior to irradiation. They were also absent in solutions of hematoporphyrin and buffer that had been degassed to high vacuum or that had been degassed and regassed with helium or nitric oxide.

No signals were obtained from solutions of hematoporphyrin irradiated at 23°C; signals present in solutions irradiated at -20°C disappeared when the samples were warmed to 23°C.

TABLE 31

Electron spin resonance study of irradiated hematoporphyrin solutions

Solution	Radical yield, %	
	-20°C	-196°C
Buffer	0	0
Buffer + serum	0	0
Buffer + hematoporphyrin	0.86	0.24
Buffer + hematoporphyrin + ascorbic acid	0	0.24
Buffer + hematoporphyrin + cysteine	0	0.30
Buffer + hematoporphyrin + serum	0.18	0.40

The results of the experiments in which the irradiations were carried out with the samples at liquid nitrogen temperatures are also summarized in Table 31. It is apparent that a signal appears in hematoporphyrin solutions irradiated at the low temperature and that this

signal is not influenced by the addition of cysteine, ascorbic acid or serum prior to irradiation. Moreover, degassing of the samples to high vacuum or replacement of the ordinary atmosphere by helium or nitric oxide prior to irradiation was followed by electron spin signals of the same character as those under ordinary atmosphere.

The signals obtained from the samples irradiated at -196°C were about one-half the size of those from the -20°C irradiations. In addition the signals from the irradiations at the two temperature extremes were of quite different character with respect to the levels of microwave power at which they became saturated, indicating that they were derived from different molecular species (Figure 55).

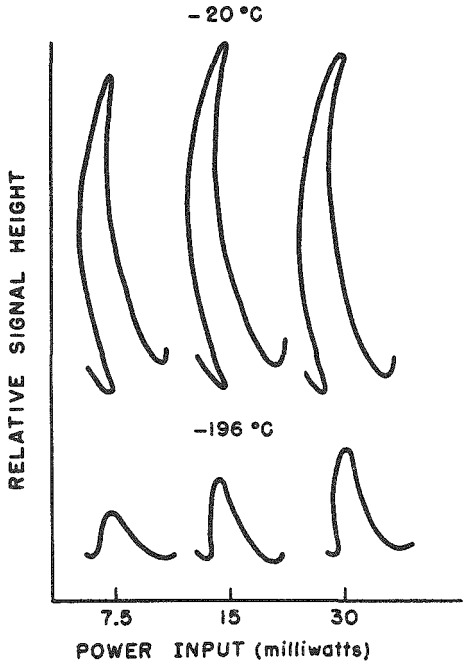

Figure 55

Comparison of ESR signals of the irradiated -196°C and -20°C systems with respect to dependence on microwave power. Line width: -196°C = 8 gauss and -20°C = 6 gauss. g =2.003 ± .001

Discussion

It seems clear that the electron spin resonance signals recorded from the solutions in the present experiments arise only as a result of irradiation and come only from hematoporphyrin. It appears that the signals arise from free radicals of hematoporphyrin.

The similarity between the conditions influencing signal production in the -20°C experiments and those influencing the course of photodynamic action are striking and suggest that the -20°C state allows the operation of a useful model system of photodynamic action. Thus, signals are produced, and photodynamic action proceeds only in the presence of oxygen, and both processes are inhibited when ascorbic acid or cysteine is introduced into the system. We interpret the oxygen dependence of the signal to indicate the presence of a free radical consisting of some kind of an association between hematoporphyrin and oxygen (oxyradical). The

failure to detect signals in the presence of ascorbic acid or cysteine is thought to be due to interaction between the reducing agent and the radical, the result of which is the loss of paramagnetic characteristics of the system.

The accumulation of radicals in measurable amounts may be readily explained in terms of decreased possibilities for reaction of radicals with other components of the system in the physical state at -20°C. The explanation of the results with cysteine, ascorbic acid and serum is not so obvious, but it appears that the -20°C state does allow interaction between these substances and radicals of hematoporphyrin. The results of the -196°C experiments, however, indicate that interactions between the critical components of the system are not possible in the physical state existing at this temperature. That the physical state of the system is an important factor was indicated in preliminary experiments in which the solvent was altered. Thus, signal production was markedly decreased when glycerol was present in 10% concentration in the usual system irradiated at -20°C. When glycerol alone was used as the solvent, a signal similar to that of the usual -196°C experiments with respect to microwave power dependence appeared after irradiation at -20°C.

It was noted above that several lines of investigation have shown that substances capable of photosensitizing oxidation are excited to the triplet state upon irradiation. It is proposed that in the present -20°C experiments, hematoporphyrin in the triplet state can combine with oxygen to form an oxyradical which may then react with the substrate according to the following scheme:

$$S + h\nu \longrightarrow {}^3S$$

$$^3S + O_2 \longrightarrow SOO\cdot$$

$$SOO\cdot + X \longrightarrow XO_2 + S$$

S, sensitizer

X, substrate

References

1. Blum, H. F. Photodynamic Action and Diseases Caused by Light. New York: Reinhold, 1941. pp. 68-75.

2. Simons, J. P. The reactions of electronically excited molecules in solution. Quart. Rev. (London) 13 3-29 (1959).

3. Lewis, G. N., and M. Kasha. Phosphorescence and the triplet state. J. Am. Chem. Soc. 66 2100-2116 (1944).

4. Lewis, G. N., M. Calvin, and M. Kasha. Photomagnetism. Determination of the paramagnetic susceptibility of a dye in its phosphorescent state. J. Chem. Phys. 17 804-812 (1949).

5. Porter, G. and M. W. The triplet state in fluid media. Proc. Roy. Soc. (London) A. 245 238-258 (1958).

TYPES OF CHROMOSOME BREAKS PRODUCED BY COLCHICINE AND X-RAY IN COLLINSIA HETEROPHYLLA

E. D. Garber*

Seeds and apical meristems of seedlings of Collinsia heterophylla, family Scrophulariaceae, were treated with colchicine in aqueous solution or lanolin. Plants with an interchange complex, the result of a reciprocal translocation of terminal segments of non-homologous chromosomes, were obtained in the progeny of self-pollinated survivors of the colchicine treatment. The complex in each of 17 independent cases occurred as a ring or chain of four chromosomes or as two bivalents. Also, the frequency of pollen mother cells with the alternate or adjacent configuration at metaphase I was approximately equal.

Seedlings at the pre-bud stage were irradiated with 50, 307, or 1,000 r (X-ray). Plants with an interchange complex were obtained in the progeny of self-pollinated survivors; 4 plants had a complex of 4 chromosomes and one plant, 6 chromosomes. A majority of the pollen mother cells had a ring complex, and the chain complex was noted in approximately 20% of the pollen mother cells; no cells displayed bivalents. One complex of 4 chromosomes occurred as an alternate or adjacent configuration at metaphase I with approximately equal frequency. The other complexes occurred more frequently (ca. 85-90%) as an alternate configuration.

The differences between reciprocal translocations resulting from colchicine treatment or irradiation are significant and indicate, probably, a difference in the site of chromosome breakage. It has been possible to propose an explanation, using the following observation and assumptions:

1. The chromosomes of C. heterophylla have only one chiasma in each arm (fact).

2. Chiasma formation is restricted to a segment of unknown length and site, probably the terminal portion of the arm (assumption).

3. Chromosome breaks produced by colchicine are restricted to the chiasma-forming segment; breaks produced by irradiation need not be restricted to the chiasma or non-chiasma-forming segments (assumption). Since 4 of the 5 interchange complexes showed a directed orientation at metaphase I, it is possible that the non-chiasma-forming segment is much longer than the chiasma-forming one or that the non-chiasma-forming segment is more easily affected by irradiation.

* Department of Botany, The University of Chicago. Work was begun while the author was a Resident Research Associate, March-June, 1959.

These observations and their explanation provide a basis for exploring the chromosomes of this species in terms of their response to irradiation with different ionizing sources and to chemical mutagens which are radiomimetic. Other potent chemical mutagens may also be included in the program. Although such an effort would entail considerable routine work, plants of this species are easily grown in relatively large numbers. Furthermore, the chromosomes are sufficiently few in number (n = 7) and reasonably large so that they may be studied under high dry magnification to permit relatively rapid screening for the presence of an interchange complex.

Current work is concerned with the production of interchange complexes in four other species of Collinsia by exposing dry seed to X-ray dosages of 10, 15, 20, and 30 x 10^3 r. The resulting data will provide a basis to determine if the observations recorded for C. heterophylla are peculiar to this species.

THE INTRACELLULAR DISTRIBUTION OF PHYTOCHROME IN CORN SEEDLINGS

S. A. Gordon

A major advance in the field of photomorphogenesis is the recent isolation of the red-far red reversible pigment phytochrome.(1) This pigment protein was salted out from alkaline corn-seedling extracts that had been centrifuged at 140k x g. Such sedimentation characteristics suggest that the complex is part of the soluble proteins of the cytoplasm.

We have found, however, that the phosphorylative capacity of Avena mitochondria is changed by red and by far-red irradiation of the plant.(2) These two radiation bands also reversibly alter the phosphorylative capacity of isolated animal mitochondria.(2) Phytochrome would thus appear to occur as a mitochondrial component. Since knowledge of the location of phytochrome in the cell could be a clue to the locus and nature of its action, the intracellular distribution of the pigment complex was investigated.

Etiolated corn seedlings, 3 days old, were dispersed in an isotonic medium. The various intracellular components indicated in Table 32 were separated by differential centrifugation. Each fraction was lysed, centrifuged at 140 k x g, and the phytochrome precipitated by 0.33 saturation with $(NH_4)_2SO_4$. The precipitates were repeptized in the buffered medium and irradiated with either red or far-red light.

TABLE 32

Red-far red absorbancy differences [$\Delta(\Delta A)$] of phytochrome from intracellular fractions of the corn seedling

Centrifugation	Description	$(\Delta A_{655} - \Delta A_{735}) \times 10^3$		
		FR vs R	R vs FR	FR vs R
10', 100 x g ↓ Supernatant	"Whole dispersion"	40	-41	35
10', 1000 x g	Plastids, nuclei wall fragments	-5	0	2
10', 4,500 x g	Plastids, nuclei	8	-6	9
30', 17,000 x g	Mitochondria	28	-33	27
30', 105,000 x g ↓ Supernatant	Microsomal / Soluble protein	1 / 12	-4 / -15	3 / 11

Organelles of plant cells intergrade in size and density,[3] and the fractions in Table 32 are to be interpreted as "containing," or "predominantly composed of," the particulates indicated. Intact nuclei were detected by the acetic-orcein technique.[4] Mitochondrial preparations were positive to Janus green; they were also able to esterify ADP to ATP,[2] a phosphorylative capacity virtually absent in the microsomal preparation. Each disrupted cellular fraction was assayed for phytochrome by using difference spectrophotometry. We assume that phytochrome is a protein complex of relatively low molecular weight that is readily peptized at neutral pH.

Tungsten filament lamps and interference filters in monochromators patterned after Withrow's[5] designs were the sources of red and far-red radiation. The far-red filter had a transmission of 50% at 735 mμ, with no radiation being detectable below 690 mμ in a Cary model 11M spectrophotometer. The red filter transmitted 75% at 655 mμ, 0.2% above 700 mμ, and cut off at 610 and 780 mμ. Incident energies were adjusted by varying the lamp voltage to yield 11 ergs/mm^2/sec at the red and 31 ergs/mm^2/sec at the far-red locus. Absorbancies of the irradiated solutions were determined in the Cary spectrophotometer using expanded scales. To maximize the differences between relatively low-order absorbancies, the phytochrome preparations were exposed to two spectral bands simultaneously. They were then rapidly compared against each other in the spectrophotometer. This procedure also permitted automatic duplication in reversal experiments. A dim green "safe light" with cut-offs at 500 and 550 mμ was used when required in the period between irradiation and determination of absorbancy differences.

Results and Discussion

Initially the method described by Butler et al.[1] for the isolation of phytochrome from corn seedlings was repeated. The preparation obtained showed a $\Delta(\Delta A_{655} - \Delta A_{735})$ of about 23×10^{-3}. This is well over the value of 5×10^{-3} that, with our instrumentation, is the minimal value for significance, and corroborates the findings of the Beltsville group. Accordingly, their procedure was modified to the procedure described above in order to measure the concentration of phytochrome in recognized organelle groupings. Table 32 gives the absorbancy differences measured after exposure of the various intracellular fractions to red and far-red radiation. It is evident that phytochrome integrity is to a large extent retained during the fractionation procedure. This is demonstrated by the ΔA at the two wavelengths, as well as the reversibility of the system. More significantly, the distribution of activity shows that the pigment complex was found both in the "soluble" protein fraction of the cytoplasm and in the mitochondrial fraction. The phytochrome concentration in the mitochondria was approximately double that found for the soluble fraction.

It is possible that phytochrome moves from the soluble cytoplasmic systems into organelle association when the pH is nonalkaline during fractionation. Alternatively, we would suggest that the alkaline conditions under which organelle integrities are not retained, and which are known to liberate proteins from bound complexes,[6] could very well free phytochrome from an organelle association. Several responses to red and far red are consistent with or support the interpretation of an *in vivo* association of phytochrome with mitochondria. These are ATP generation by mitochondria from irradiated *Avena* seedlings,[2] phosphorylation by irradiated liver mitochondria,[2] and the activation of phosphate esterification in lettuce seed.[7] The response of lettuce seed can be correlated with the photomorphogenic effects of the red spectral region on germination.

Summary

Intracellular fractions of the corn seedlings were obtained by differential centrifugation. These were analyzed for phytochrome. The pigment protein was found associated with mitochondria as well as in the nonparticulate fraction. This association is discussed briefly in relation to the control of phosphorylation by red and far-red radiation.

References

1. Butler, W. L., K. H. Norris, H. W. Siegelman and S. B. Hendricks. Detection, assay, and preliminary purification of the pigment controlling photoresponsive development of plants. Proc. Natl. Acad. Sci. 45 1703-1708 (1959).

2. Gordon, S. A., and K. Surrey. Red and far-red action on oxidative phosphorylation. Radiation Research 12 325-339 (1960).

3. Goddard, D. R., and H. A. Stafford. Localization of enzymes in the cells of higher plants. Ann. Rev. Plant. Physiol. 5 115-132 (1954).

4. Tjio, J. H., and T. T. Puck. Genetics of somatic mammalian cells. II. Chromosomal constitution of cells in tissue culture. J. Exptl. Med. 108 259-268 (1958).

5. Withrow, R. B. An interference-filter monochromator system for the irradiation of biological material Plant Physiol. 32 355-360 (1957).

6. Blish, M. J., and R. M. Sandstedt. The nature and identity of wheat glutenin. J. Biol. Chem. 85 195-206 (1929).

7. Gordon, S. A., and K. Surrey. Phosphorylation and red-spectrum photomorphogenesis. In Proc. Symposium on Effects of Ionizing Radiation on Seeds, Karlsruhe, Germany. Intern. Atomic Energy Agency, 1960. In press.

A NEW PATHWAY OF AUXIN BIOGENESIS

S. A. Gordon and L. G. Paleg

The radiosensitivity of auxin biogenesis in the plant has emphasized the lack of exact knowledge about the biochemistry involved. It is quite probable that the endogenous auxin of hormonal function in the plant is indoleacetic acid (IAA), with tryptophan serving as its primary precursor. However, evaluation of IAA biosynthesis in vitro is impeded by the low yields usually obtained. We found that the addition of certain phenols to the enzymatic medium resulted in 10-fold increases in IAA concentration. This led to the discovery of what appears to be a new pathway for biosynthesis of the auxin: the degradation of tryptophan to IAA via polyphenolase systems. More specifically, dihydroxy phenols are oxidized to their quinones; the quinones deaminate tryptophan to indolepyruvic acid, which in turn breaks down to IAA. Experiments upon which this pathway is postulated are summarized below.

1. With either fresh or lyophilized enzyme preparations of the oat coleoptile, or of the seedling of the sunflower or mung bean, the conversion of tryptophan to IAA is enhanced 10 to 30 times in the presence of catechol.

2. This enhancement is not caused by a reduction in IAA inactivation; the addition of catechol to the enzyme in suitable media has no effect on the recovery of auxin added to the medium.

3. Other phenols can replace catechol, with similar orders of activity: 3-nitrocatechol, protocatechuic, caffeic and chlorogenic acids, and phenole; pyrogallol and DOPA are less active; tyrosine, resorcinol and o-nitrophenol are inactive. The vicinal dihydroxyphenol structure is apparently required since only such phenols (or compounds readily converted to that structure) possess appreciable activity.

4. So-called "structural" phenolase inhibitors, nitro-monophenols, do not inhibit the action of either catechol or phenole on tryptophan. However, 2,4-dinitrophenol in equimolar concentration does inhibit.

5. Auxin formation by the catechol system is inhibited by reducing and chelating agents - strongly by ascorbate cysteine and DIECA, weakly by EDTA and o-phenanthroline, and not by α,α-dipyridyl or azide.

6. The action of the mung bean enzyme in the tryptophan-catechol system can be replaced by mushroom tyrosinase. A polyphenolase function of the mung bean enzyme may be inferred.

7. A polyphenolase action of the enzyme would use O_2 as an acceptor and form a quinone as the first product. We find that the action of the enzyme-catechol system is inhibited in an N_2 atmosphere, and that the formation of IAA from tryptophan can also be accomplished by contact of the amino acid with ethereal solutions of freshly synthesized o- and p-benzoquinone.

8. A defined model system for the conversion of tryptophan to IAA was achieved. Catechol was allowed to react with proline in the presence of ferricyanide. The reddish pigment so produced formed IAA upon the subsequent addition of tryptophan.

9. The likelihood that indoleacetaldehyde is an intermediate in the degradation of tryptophan to IAA by the catechol system was explored. a) We find that IAA is not produced when tryptamine is added to the catechol-catecholase system. b) Indoleacetaldehyde was synthesized and added to the catecholase system. No IAA was formed. c) The aldehyde-specific reagent dimedone was added to the tryptophan-catechol catecholase mixture. No effect on the yield of IAA was apparent. It may be concluded that indoleacetaldehyde is not an intermediate in this path of auxin synthesis.

10. Since tryptamine and indoleacetaldehyde were apparently not intermediates, the oxidation of tryptophan via the keto acid route was then considered. Crystalline indole-3-pyruvic acid, synthesized in this Division by J. H. Pomeroy and C. Craig, was chromatographed in an alkaline solvent system. Parallel runs were made of the acid fraction of the tryptophan-catechol incubation mixture. Five of the decomposition spots characteristic of indolepyruvate were found on the chromatograms of the tryptophan-catechol reaction.

The above evidence that the keto acid is an intermediate in the phenolic degradation of tryptophan was strengthened by the following experiment. Tryptophan-3-C^{14} was used as a substrate for the catechol-catecholase system. Its acid plus neutral fraction was passed through a cellulose column, with a parallel column of the synthetic indolepyruvate as a marker. The solvent was removed from the appropriate eluate of the C^{14}-labeled incubation mixture. After the addition of unlabeled crystalline indolepyruvic acid to the residue, the mixture was converted to the p-nitrophenylhydrazone. This was recrystalized four times. The last three crystalline products had a constant specific activity.

We consider that the above series of observations on IAA formation are consistent with the mechanisms now proposed for the oxidative deamination of amino acids by the phenolase complex.(1) Briefly, o-dihydroxyphenols are oxidized by phenolase to the corresponding o-quinone. Where molecular oxygen is the acceptor, low H^+ concentrations promote the reaction rate. The quinone condenses spontaneously with amino acids to form a p-substituted reddish pigment of the amino quinone class. This primary condensation

product reacts again with free amino acid to form the o-quinonimine as a second condensation product. The quinonimine spontaneously rearranges, hydrolizes and reoxidizes; in this process a keto acid is liberated, presumably from the o-bound amino acid. If tryptophan is the amino acid, we have shown that the keto acid is probably indolepyruvate. This keto acid is unstable and breaks down spontaneously in solution to IAA.

Does the preceding scheme represent the pathway of normal auxin biogenesis? It is relevant that the distribution of biological activity on chromatograms of the acid fraction from the reaction of tryptophan and the mung bean enzyme is qualitatively similar to the distribution obtained from the reaction of tryptophan, catechol, and enzyme. However, the above pathway may not be followed in normal tissue because of the following two considerations.

First, there are indications that the phenolases of plants are associated with cellular particulates.[2] Yet it has been shown[3] that none of the essentially intact cell organelles produce IAA from tryptophan in the absence of catechol; such activity is a "soluble" component of the cytoplasm. Though the above considerations can hardly be thought of as convincing evidence, it is highly probable that the phenols and phenoloxidases are spatially separated in intact cells. The tryptophan-phenolase-phenol reaction could thus be considered as a latent, potentially operative mechanism for auxin formation in the plant. It may be suggested that lysis occurring upon the wounding of plant tissue, a process wherein activation of the phenolase complex takes place, produces abnormal quantities of growth hormone via the phenol-tryptophan reaction. Callus and gall formation could be histogenic consequences of such hyperauxiny, and there would be no need to postulate the function of a second wound hormone such as traumatin in the morphogenesis of wound regeneration

Second, responses to ionizing radiation indicate a nonfunction of this phenolase action in normal tissues. Normal auxin biogenesis is highly sensitive in vivo to X- and γ-radiation. This radiosensitivity is rapidly manifest in a reduced level of the free hormone, in alterations of morphological phenomena dependent upon hormonal concentration, and in the activity of the soluble enzyme converting tryptophan to auxin. In current experiments we have found that the phenolase-mediated conversion of tryptophan to IAA is unimpaired in preparations made from seedlings immediately after exposure to single X-ray doses of 5 kr.

It is quite likely that the phenolase complex is activated by the disruptions in cellular integrity that result from chronic irradiation. A consequent hyperauxiny could well be the basis for the hyperplasia and neoplasms that are commonly observed in such irradiated plants.[4]

References

1. Trautner, E. M., and E. A. Roberts. The chemical mechanism of the oxidation deamination of amino acids by catechol and polyphenolase. Austral. J. Sci. Research B 3 356-380 (1950).

2. Bonner, W. D. Soluble oxidases and their functions. Ann. Rev. Plant Physiol. 8 427-452 (1957).

3. Gordon, S. A. Intracellular localization of the tryptophan-indoleacetate enzyme system Plant Physiol. 33 23-27 (1958).

4. Gunckel, J. E., and A. H. Sparrow. Aberrant growth in plants induced by ionizing radiation. Brookhaven Symposium VI, BNL-258 (1958). pp. 252-279.

PHOTOPERIODIC RESPONSES OF SEQUOIA GIGANTEA SEEDLINGS

John Skok

Photoperiodic responses of numerous woody species are well known, but in general, photoperiodic information on tree species is considerably less abundant than that for herbaceous plants. No information in this regard has been reported for Sequoia gigantea.

An observation in this laboratory of a very unusual occurrence involving cone-formation in a single 11-month old Sequoia seedling (Figure 56) prompted further experiments. Plantings were maintained under 8, 12, 14, 16 and 18-hr photoperiods, but fruiting was not again obtained. Photoperiod apparently does not influence cone formation in young seedlings, and no explanation can be offered for this single observed case. The experiments did, however, illustrate that growth responses are markedly influenced by photoperiod (Figure 57).

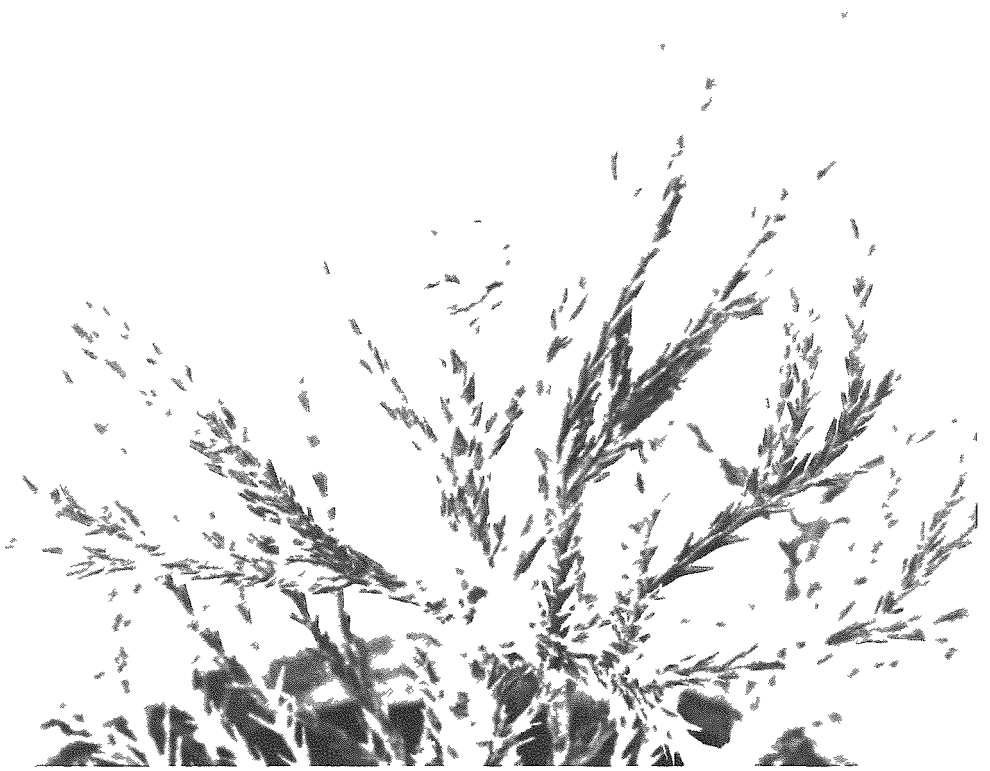

Figure 56. Sequoia gigantea seedling 11.5 months from sowing, bearing a terminal female cone.

Figure 57. Effect of photoperiod on Sequoia gigantea seedlings, 18.7 months from sowing: 1, 8 hr, 2, 12 hr, 3, 16 hr, and 4, 18 hr.

Growth, as measured by main axis heights, number and total length of primary branches and stem diameters, was depressed by short photoperiods and stimulated by long photoperiods, while intermediate photoperiods produced intermediate amounts of growth. The longest effective photoperiod (sunrise to sunset period plus civil twilight) that prevails during the long days of summer in the natural habitat range for Sequoia is about 16 hr. Under the presently employed experimental conditions, the 16-hr plants made relatively vigorous growth, but it is of interest to note that it was possible to increase this growth rate measurably by extending the photoperiod to 18 hr.

The abundant data on photoperiodic responses that have become available over the past forty some years bear out the conclusion that most, if not all, plants respond somehow or other to day length. Even so-called day-neutral plants, plants that are not specifically photoperiod-dependent for flowering, generally show some growth effects. It is of interest that Sequoia gigantea is not an exception in this respect. This is of further interest in that this species has been restricted to an extremely narrow and isolated natural habitat for several thousands of years. Photoperiod, obviously, has had no apparent ecological impact that resulted in widening its distribution even though the species is responsive to photoperiod.

Since photoperiod has such marked effects on seedling vigor, it would appear that reseeding and maintenance of the species might be influenced to a degree by latitudinal distribution. The more northerly latitudes of longer summer days would be conducive to vigorous seedling growth provided the winter temperatures remained within tolerance range. On this basis it could be expected that certain sites in Washington, Oregon and British Columbia might provide favorable conditions for successful establishement of the species.

A DIFFICULTY WITH IODOMETRIC TECHNIQUES APPLIED TO CERTAIN PEROXIDE MIXTURES*

R. N. Feinstein

In classical iodometry, an excess of KI is added to the acidified sample, together with a drop of 5% ammonium molybdate, and the free iodine released is titrated with standard $Na_2S_2O_3$. This technique was used with complete satisfaction in the analysis of pure solutions of H_2O_2, disuccinoyl peroxide (DSP), or quinone, but considerable difficulty has been encountered in applying it to mixtures of H_2O_2 or DSP with quinone, or to an H_2O_2-hydroquinone-copper system.

The difficulty takes the form of a delayed, recurrent end point. After an apparently final end point is reached, if the solution remains at room temperature for a time, more free iodine appears. An auxiliary titration produces another end point, and the solution will then again recolor. Eventually no further recoloration appears, and the total of the titrations is then approximately stoichiometric. (A loss of several percent is generally encountered in such mixtures.) The recurrent end point cannot be avoided merely by permitting a longer period before the first titration; the free iodine must be reduced before more free iodine appears.

Tables 33 and 34 illustrate the extent of the recurrent end point. In these determinations, 5-ml aliquots of (approx) 0.01 M H_2O_2 (or DSP) and (approx.) 0.01 M quinone were titrated separately and combined. In each case, 2 ml of 1 N H_2SO_4 was added

TABLE 33

Iodometric titration of H_2O_2, quinone, and mixture

Substance	$Na_2S_2O_3$ titrated, meq			% loss
	Initial	Auxiliary	Total	
H_2O_2 alone	0.105	0.000	0.105	
Quinone alone	0.096	0.001	0.097	
H_2O_2 plus quinone	0.186	0.011	0.197	2.5

* The experiments here recorded were performed during the tenure of a Guggenheim Fellowship at the Institut du Radium, Paris, France.

TABLE 34

Iodometric titration of DSP, quinone, and mixture

Substance	$Na_2S_2O_3$ titrated, meq			% loss
	Initial	Auxiliary	Total	
DSP alone	0.095	0.000	0.095	
Quinone alone	0.082	0.000	0.082	
DSP plus quinone	0.146	0.019	0.165	6.8

An attempt was made to determine the factors influencing the extent of the recurrence. One factor of proven importance is the concentration of acid; less recurrence is observed in the presence of more concentrated acid. This is illustrated in Table 35, where the negligible effect of acid on the titration of H_2O_2 alone is compared with its effect on the titration of a mixture of H_2O_2, hydroquinone, and $CuSO_4$.

TABLE 35

Effect of acid on end point recurrence

Substance	Final normality of H_2SO_4	$Na_2S_2O_3$ titrated, meq		
		Initial	Auxiliary	Total
H_2O_2	0.1	0.110	0.001	0.111
H_2O_2	0.2	0.109	0.001	0.110
H_2O_2	1.0	0.109	0.001	0.110
H_2O_2	4.0	0.110	0.001	0.111
H_2O_2-hydroquinone-$CuSO_4$	0.1	0.100	0.008	0.108
H_2O_2-hydroquinone-$CuSO_4$	0.2	0.103	0.006	0.109
H_2O_2-hydroquinone-$CuSO_4$	0.5	0.107	0.003	0.110
H_2O_2-hydroquinone-$CuSO_4$	1.0	0.108	0.001	0.109
H_2O_2-hydroquinone-$CuSO_4$	1.5	0.108	0.001	0.109
H_2O_2-hydroquinone-$CuSO_4$	2.0	0.109	0.001	0.110
H_2O_2-hydroquinone-$CuSO_4$	4.0	0.109	0.001	0.110

Other than acid concentration, no factor has been observed that is of importance in emphasizing or diminishing the end-point recurrence. Among the factors tested were the relative and absolute amounts of all components; length of standing at each stage of the reaction; and order of addition of the reagents; and temperature.

This sort of recurrent, sluggish end point in iodometry has been mentioned before in the literature, particularly by Everett and Minkoff.[1] These authors noted the same difficulty in titrating mixtures of H_2O_2 and hydroperoxides. They offered no explanation for their phenomenon.

Reference

1. Everett, A. J., and G. J. Minkoff. The analysis of combustion products. III. The effect of hydrogen peroxide on the reaction of potassium permanganate with organic peroxides in acid solution. Anal. Chim. Acta 11 48-53 (1954).

PRELIMINARY STUDY OF CHEMICAL TESTS TO MEASURE THE REACTIVITY OF PEROXIDES*

R. N. Feinstein

This project was undertaken in the hope of finding a test that would classify peroxides according to chemical reactivity in the same order as their relative biological activity, with particular reference to their radiomimetic effect on the transforming factor. It was hoped that such a test would permit prediction of radiomimicry.

Three peroxides were studied in each of three tests. The peroxides were: (a) H_2O_2, (b) disuccinoyl peroxide (DSP), (kindly provided by P. Demerseman), and (c) cumene hydroperoxide (CHP), a commercial sample from the Hercules Powder Company and stated to assay 73.2% CHP. These three were selected because they have been tested by Latarjet et al.(1) for their effect on the transforming factor, in which case their order of activity was found to be DSP $>>>$ H_2O_2 $\gg$ CHP.

The three reactions tested were: (a) oxidation of hydroquinone, (b) reaction with the anion exchange resin Dowex 1, and (c) release of free iodine from solutions of KI. These three tests are discussed in the following sections.

Oxidation of Hydroquinone

This test had been investigated, in the case of DSP only, by Demerseman and Mathieu,(2) who had found that, under certain circumstances, DSP oxidized hydroquinone to quinone, as measured by increased absorption at 2500 A. The necessary "circumstance," unknown at that time, proved to be a need for metal catalyst, either copper or iron. (It was ultimately demonstrated that as little as 10^{-11} M copper ion is detectably catalytic, which makes this test an extremely delicate means of detection of these ions.) When an attempt was made to apply this test to H_2O_2 or CHP, however, it was found that while color formation indicated that a reaction was indeed proceeding, there was very little, if any, increase in absorption at 2500 A, hence little, if any, formation of quinone. The following remarks, with the exception of Table 39, apply to the formation of a visible pink color.

The reaction is catalyzed most strongly by iron (both ferrous and ferric), slightly less strongly by cupric ion, very slightly by zinc, and not at all by Mg, Mn, Ca, or Co. The simultaneous presence of Mn, at 10-fold

* These experiments were performed during tenure of a Guggenheim Fellowship at the Institut du Radium, Paris.

the concentration of the other metals, is without effect on the rate of catalysis by iron, copper, or zinc. The catalysis is prevented, or halted, by addition of ethylenediaminetetraacetic acid (EDTA) or H_2SO_4. EDTA in twice the molar concentration of the metal is completely effective. (Presumably stoichiometric amounts of EDTA would also be effective, but double amounts were always used to assure an excess.) Addition of an excess of copper to the EDTA-arrested reaction permits the reaction to proceed again; addition of excess copper is without effect on the H_2SO_4-terminated reaction.

As shown by Ingraham[3] in the case of the oxidation of catechol by H_2O_2 in the presence of Cu, the reaction rate is critically dependent on concentration of H_2O_2, hydroquinone, and metal, as well as on pH and temperature. The effects of copper concentration, temperature, and pH are shown in Tables 36, 37, and 38, respectively.

TABLE 36

Effect of copper concentration on the H_2O_2-hydroquinone reaction rate

(Temperature, 20°C; pH, 4.5)

$CuSO_4$ concentration, M	Time to initial color
No copper	ca 90 min
10^{-6}	31 min
10^{-5}	7 min
10^{-4}	40 sec
10^{-3}	10 sec

TABLE 37

Effect of temperature on the H_2O_2-hydroquinone reaction rate

(pH, 4.5)

Temperature, °C	Time to initial color appearance, sec	
	2×10^{-5} M $CuSO_4$	10^{-5} M $CuSO_4$
20	150	420
25	90	270
30	75	160
35	60	105
40		75
45		55
50		40

TABLE 38

Effect of pH on the H_2O_2-hydroquinone reaction rate

(Temperature, 20°C; $CuSO_4$, 10^{-5} M)

pH	Time to initial color, min
3.5	50
4.1	20
4.5	7.5
5.2	5

Table 39 indicates the rate of quinone formation (absorption at 2500 A) from hydroquinone by DSP, H_2O_2, and CHP, each in the presence of copper catalyst. It is apparent that DSP rapidly produces quinone, while H_2O_2 and CHP produce very little, if any, quinone. (In another experiment, not shown, there was no indication of quinone formation from hydroquinone plus H_2O_2 plus Cu.)

TABLE 39

Rate of formation of quinone from hydroquinone-peroxide-copper

Peroxide, 10^{-3} M; $CuSO_4$, 4×10^{-6} M; hydroquinone, 2×10^{-3} M. Temperature 20°C, pH, 4.5. Reaction was stopped when desired by addition of equal volume of 4×10^{-5} M EDTA. For spectrophotometric reading, these solutions were then diluted 20-fold further with 2×10^{-5} M EDTA.

Time, min	Optical density at 2500 A		
	DSP	H_2O_2	CHP
0	0.006	0.025	0.013
15	0.206	0.029	0.022
30	0.311	0.036	0.025
60	0.405	0.040	0.036
120	0.436	0.045	0.051

Despite the lack of quinone formation from hydroquinone by H_2O_2 or CHP, it is obvious that some reaction is taking place, for all three peroxides produce visible colors. Interestingly, the colors are not identical. H_2O_2 produces a pink color, DSP a yellow, and CHP a green. If the H_2O_2 product is acidified, the pink becomes yellow but still shows no absorption at 2500 A.

The relative rate of initial appearance of a given color under varying circumstances may be estimated visually with some accuracy. The estimation of relative rates of initial appearance of different colors must be

considered much less accurate. Only if differences in rate are considerable may they be considered significant. An attempt has been made to estimate in this manner the relative rate of appearance of color under the influence of DSP vs H_2O_2 vs CHP. This was done at a variety of copper concentrations and at a variety of temperatures. The results are shown in Tables 40 and 41. Despite the imprecise technique employed, it seems clear that H_2O_2 produces color appreciably more rapidly than do DSP or CHP. Although there is no striking difference between the latter two, I would suggest that the data of Table 41 be given somewhat more credence than those of Table 40 since some difficulty was experienced in precise reproduction of the data of Table 40. On this basis, then, one is inclined to argue that in this system, the CHP actually shows slightly greater activity than does the DSP. Certainly the DSP here does not show its more customary considerable advantage over CHP.

TABLE 40

Effect of copper concentration on relative rate of color production from hydroquinone-peroxide-copper

Peroxide, 2×10^{-4} M; hydroquinone, 0.01 M; pH 4.5, 20°C.

$CuSO_4$ concentration, M	DSP	H_2O_2	CHP
10^{-5}	+*	+	0
10^{-4}	+	++	+
10^{-3}	?	++	++

* ++, color detectable is less than 10 min.
+, color not detectable in 10 min but detectable within 30 min.
0, color not detectable within 30 min.

TABLE 41

Effect of temperature on relative rate of color production from hydroquinone-peroxide-copper

Peroxide, 2×10^{-4} M; hydroquinone, 0.01 M; $CuSO_4$, 10^{-4} M; pH 4.5

Temperature, °C	DSP	H_2O_2	CHP
21	+1*	+3	+1
35	+4	+5	+5
50	+6	+6	+6

*+6 Color within 15 sec.
+5 Color in 16-30 sec.
+4 Color in 31-60 sec.
+3 Color in 61-90 sec.
+2 Color in 91-300 sec.
+1 Color detectable only after 300 sec.

It is interesting that Vermeil and Salomon,[4] irradiating a simple solution of hydroquinone with X-rays, obtained a colored product with properties strikingly similar to those of the H_2O_2-hydroquinone-copper product: pink until acidified, then yellow, and with a very similar absorption spectrum in the visible and ultraviolet range. The fact that X-radiation in the absence of metal catalyst produced what appears to be the same product as that produced by H_2O_2 and copper or iron, makes it appear likely that hydroxyl radicals are involved. This sort of Fenton's reaction is well discussed by Haber and Weiss.[5] It is possible, in fact, that the reason for the decreased activity of DSP in the formation of colored products lies in the relative rate of production of hydroxyl radicals and of other oxidizing radicals. It at least seems evident that the formation of quinone from hydroquinone by DSP and copper is not mediated by hydroxyl radicals.

Reaction of Peroxides with Anion Exchange Resin

The reaction with Dowex 1 (hydroxyl form) was suggested by some earlier work,[6] in which ether was freed of peroxides by contact with the resin. It was considered at that time that the reaction was purely one of anion exchange. However, in the present studies it has not been possible to recover the peroxide (H_2O_2 or DSP) from the resin, and it is tentatively concluded that the reaction is <u>not</u> simply an exchange of anions. The exact nature of the reaction remains unknown.

Analyses for H_2O_2 and DSP were carried out by classical iodometry, in which the peroxide is permitted to release its equivalent of free iodine from KI solutions, the free iodine then being titrated with standard thiosulfate (here used 0.005 $\underline{N}$ $Na_2S_2O_3$). This technique, however, is not suitable for the measurement of CHP because of its low reactivity in this respect (cf. following section). A modification of the method of Hochanadel[7] was therefore employed. Hochanadel's reagent was mixed with an equal volume of the CHP-containing solution, and the mixture was permitted to stand at room temperature for 24 hr before titrating with standard $Na_2S_2O_3$. Occasionally a slight recurrence of the end point was observed, and a small auxiliary titration was required after several hours more. This technique was reproducible within perhaps 5%.

The amount of a given peroxide which will be retained by the resin is dependent both upon the concentration of the peroxide solution applied and upon its volume, the volume being of lesser importance in this connection. These statements are verified by the data of Tables 42-46.

It will be noted from these tables that it was necessary to vary the ratio of peroxide to resin considerably for the various peroxides, in order to have accurately measurable recoveries. Despite this fact, and the additional fact of variation in retention with variation in concentration and volume of peroxide, it is evident that the three peroxides are distinctly different quantitatively with regard to their reaction with the ion exchange resin.

TABLE 42

Effect of concentration of DSP on retention by Dowex 1

Resin and peroxide were mixed mechanically for 5 min, then filtered through small circles of fast paper. Aliquots of the filtrates were analyzed for peroxide as stated in the text. Peroxide "retained by resin" was calculated by difference.

Weight of resin, g	Conc. DSP, M	DSP applied to resin, ml	DSP applied to resin, meq	DSP remaining in soln., meq	DSP retained by resin, meq
0.200	0.03	10.0	0.600	0.391	0.209
0.200	0.02	10.0	0.400	0.232	0.168
0.200	0.01	10.0	0.200	0.064	0.136
0.200	0.005	10.0	0.100	0.005	0.095

TABLE 43

Effect of concentration of H_2O_2 on retention by Dowex 1

Weight of resin, g	Conc H_2O_2, M	H_2O_2 applied to resin, ml	H_2O_2 applied to resin, meq	H_2O_2 remaining in soln., meq	H_2O_2 retained by resin, meq
1.000	0.101	10.0	2.02	1.69	0.33
1.000	0.0808	10.0	1.62	1.36	0.26
1.000	0.0606	10.0	1.21	1.03	0.18
1.000	0.0404	10.0	0.81	0.69	0.12
1.000	0.0202	10.0	0.40	0.36	0.04

TABLE 44

Effect of volume of DSP on retention by Dowex 1

Weight of resin, g	Conc. DSP, N	DSP applied to resin, ml	DSP applied to resin, meq	DSP remaining in soln., meq	DSP retained by resin, meq
0.200	0.00396	25	0.990	0.774	0.216
0.200	0.00396	20	0.792	0.588	0.204
0.200	0.00396	15	0.594	0.398	0.196
0.200	0.00396	10	0.396	0.215	0.181
0.200	0.00396	5	0.198	0.040	0.158

TABLE 45

Effect of volume of H_2O_2 on retention by Dowex 1

Weight of resin, g	Conc. H_2O_2, N	H_2O_2 applied to resin, ml	H_2O_2 applied to resin, meq	H_2O_2 remaining in soln., meq	H_2O_2 retained by resin, meq
1.000	0.00294	50	0.147	0.140	0.007
1.000	0.00294	40	0.117	0.109	0.008
1.000	0.00294	30	0.088	0.080	0.008
1.000	0.00294	20	0.059	0.052	0.007

TABLE 4b

Effect of volume of CHP on retention by Dowex 1

Weight of resin, g	Conc. CHP, N	CHP applied to resin, ml	CHP applied to resin, meq	CHP remaining in soln., meq	CHP retained by resin, meq
1.000	0.00291	50	0.146	0.096	0.050
1.000	0.00291	40	0.116	0.071	0.045
1.000	0.00291	30	0.087	0.047	0.040
1.000	0.00291	20	0.058	0.026	0.032

The approximate order of magnitude of reaction may be stated, from the tables above, to be:

DSP 1.0 meq/g resin (using ca. 4×10^{-2} N DSP)

H_2O_2 0.04 meq/g resin (using ca. 4×10^{-2} N H_2O_2)

H_2O_2 0.008 meq/g resin (using ca. 3×10^{-3} N H_2O_2)

CHP 0.04 meq/g resin (using ca. 3×10^{-3} N CHP).

Hydrogen peroxide may thus conveniently be used as a standard by which to compare other peroxides. It avoids, on the one hand, the difficulty of limited solubility of such peroxides as CHP, and it avoids on the other hand, the extremely high reactivity of the DSP. For a thorough-going mathematical analysis of these systems, complete with equilibrium constants, more data would be necessary. However, on the basis of the figures given just above, one may hazard the very crude estimate that DSP is perhaps 25 times as active as H_2O_2, while CHP is perhaps five times as active as H_2O_2. The resulting order of activity then is DSP > CHP > H_2O_2.

Release of Free Iodine from Solutions of KI by Peroxides

Measurement of the rate of release of iodine from aqueous solutions of KI suffers from two important disadvantages: (1) it has not been found possible to halt the reaction at any given point for measurement, and (2) the range of reaction rate is so great as to make it difficult to devise one single technique which will be applicable to all peroxides. Generally, an acid reaction is necessary for the release of iodine from KI. However, an active agent such as DSP will very quickly, essentially instantaneously, release its equivalent of iodine in the presence of buffers of pH as high as 9 or 10. An inactive agent such as CHP only slowly releases iodine even in the presence of 1 N H_2SO_4, and allowance must here be made for iodine which is released due to oxidation by the H_2SO_4 itself. A peroxide of intermediate activity, such as H_2O_2, will release iodine from KI at convenient, reproducible rates, the exact rate being dependent upon the pH, temperature, and concentration of the peroxide. For these reasons, most of the following experiments have been carried out with hydrogen peroxide as oxidizing agent.

The general principle of the following experiments has been to prepare two solutions, the first containing 1.0 ml of the peroxide together with 10.0 ml of acid or buffer, the second containing 1.0 ml of freshly prepared 10% KI. The reaction is initiated by mixing the two solutions, and an end point is considered to be that time at which the color reaches that of a standard ferric chloride solution. Since the ultimate color obtainable depends on the initial concentration of peroxide, a series of standards was necessary.

The effect of temperature is shown in Table 47. The same effect of temperature has been observed at other pH values and with other concentrations of peroxide.

TABLE 47

Effect of temperature on iodine release from KI by H_2O_2

Initial conc. H_2O_2, 0.05 M; acid source, 0.1 N H_2SO_4.
End point comparison color: 10^{-3} M $FeCl_3$.

Temperature, °C	Time to end point, sec
20	135
35	60
50	30
65	15

To obtain the data of Table 48, the temperature was maintained at 20°C, and the concentration of H_2SO_4 was varied. The end point was matched with the color of 5×10^{-4} M $FeCl_3$. The concentration of H_2SO_4 shown is that before mixing with the peroxide and KI. H_2O_2 was 0.01 M before mixing with the other components.

TABLE 48

Effect of acid concentration on release of iodine from KI by H_2O_2

Concentration of H_2O_2, 0.01 M; concentrations of H_2O_2 and of H_2SO_4 stated are before mixing with other components.
End point comparison color: 5×10^{-4} M $FeCl_3$.

Original conc. of H_2SO_4, N	Time to end point, min
0.01	15
0.025	14
0.05	10
0.10	7
0.25	4
0.50	2
1.00	1

Table 49 demonstrates that the reaction rate is also critically dependent upon the concentration of the peroxide. In this experiment, despite the variation in H_2O_2 concentration, the same end point was of course employed, namely that of 2×10^{-4} M $FeCl_3$.

TABLE 49

Effect of H_2O_2 concentration on release of iodine from KI

Acid source, M/10 phosphate buffer, pH 2.1; temperature, 30°C.
End point comparison color: 2×10^{-4} M $FeCl_3$ throughout.

Original conc. of H_2O_2, M	Time to end point, sec
0.005	630
0.010	380
0.025	135
0.050	75
0.100	40

The following miscellaneous observations are of interest in connection with this test:

(a) Copper and iron, in contrast to their behavior in the hydroquinone system, do not accelerate the rate of release of iodine from KI solutions by H_2O_2. In fact, $FeSO_4$ solutions appear to diminish the rate. This, however, is due to the strong catalatic effect of iron on H_2O_2 solutions. Mixtures of H_2O_2 and $FeSO_4$, initially clear and colorless, rapidly become turbid from oxygen formation and yellow from formation of (presumably) $Fe_2(SO_4)_3$, which precipitates out. Copper has much less catalatic effect, although some bubbles of gas can be observed. At a ratio as high as one mole of $CuSO_4$ to fifty moles of H_2O_2, no effect is observed on the rate of release of iodine from KI.

(b) In measuring the effect of CHP on the release of iodine from KI, the following four mixtures were prepared and maintained at 20°C:

Mixture	Time to reach end point
1 ml 10^{-3} M CHP + 10 ml 1 N H_2SO_4 + 1 ml 10% KI	19 min
1 ml H_2O + 10 ml 1 N H_2SO_4 + 1 ml 10% KI	32 min
1 ml 10^{-3} M CHP + 10 ml 0.1 M H_3PO_4 + 1 ml 10% KI	120 min
1 ml H_2O + 10 ml 0.1 M H_3PO_4 + 1 ml 10% KI	No color within 18 hr

It is thus evident that CHP does have an effect on this system, that this effect is extremely slight, and that 1 N H_2SO_4 by itself (but not 0.1 M H_3PO_4 by itself) can release I_2 from KI solution.

(c) DSP, even at concentrations as low as 10^{-3} M, and even in the presence of M/10 phosphate buffer at pH ca. 9.5, essentially instantaneously releases its equivalent of iodine from KI. Cooling to 0°C has no detectable effect on the speed of the reaction.

Summary and Conclusions

The following very briefly summarizes the over-all conclusions to be drawn from the foregoing experiments:

1. In the hydroquinone-peroxide system, DSP behaves qualitatively differently from H_2O_2 and CHP, quinone being the primary product in the former case but being a very minor (if indeed existent) product in the latter cases. In all cases, metal catalyst (Cu or Fe) is required. If one observes only visible formation of color, one concludes that the order of reactivity is: $H_2O_2 > CHP > DSP$.

2. In the reaction of peroxides with Dowex 1, the order of reactivity is evidently DSP $\gg$ CHP $>$ H_2O_2.

3. In the release of free iodine from KI by peroxide, the order of reactivity is DSP $\gg H_2O_2 \gg$ CHP.

In the biological test of these three peroxides on the transforming factor,[1] it was found that DSP is about 10^4 times as active as H_2O_2, while CHP was essentially inactive. It, therefore, appears that the third test above, i.e , release of free iodine from KI, is the only one of the three which puts these compounds in the same order as their biological effectiveness.

I would like to record here my very considerable gratitude to Doctors Latarjet, Demerseman, Mathieu, Royer, and Ekert, for assistance in ways too numerous to detail - discussion, supply, instruction, and others.

References

1. Latarjet, R., N. Rebeyrotte, and P. Demerseman. Action of organic peroxides on a transforming factor of Pneumococcus. In Organic Peroxides in Radiobiology, London: Pergamon Press, 1958.

2. Demerseman, P., and A Mathieu. Personal communication.

3. Ingraham, L. L. Model copper oxidases: the cupric ion-catalyzed hydrogen peroxide oxidation of pyrocatechol. Arch. Biochem. Biophys. 81 309-318 (1959).

4. Vermeil, C., and L. Salomon. Action of X-rays (18.5 kev) on aqueous solutions of benzoquinone and hydroquinone. Compt. rend. Acad. Sci. 249 268-270 (1959).

5. Haber, F., and J. Weiss. The catalytic decomposition of hydrogen peroxide by iron salts. Proc. Roy. Soc. A147 332-351 (1934).

6. Feinstein, R. N. Simple method for removal of peroxides from diethyl ether. J. Org. Chem. 24 1172-1173 (1959).

7. Hochanadel, C. J. Effects of cobalt gamma-radiation on water and aqueous solutions. J. Phys. Chem. 56 587-594 (1952).

ATTEMPTS AT CANCER THERAPY WITH CATALASE

R. N. Feinstein and Marilyn Vetter

In a recent monograph,[1] Puig Muset presented arguments, both theoretical and experimental, for the possibility that the enzyme catalase should serve as an effective therapeutic agent against malignancies. Actually, this concept runs counter to the more prevalent view that if total body catalase could somehow be eliminated, endogenous or exogenous hydrogen peroxide (fed, injected, produced enzymatically, or formed by ionizing radiation) would be therapeutically effective. Warburg and his group have most effectively advocated this latter concept.[2-4]

However, in correspondence with Puig Muset, it was agreed to test his concept on animal tumors, the studies to be preliminary in nature and to be expanded only if results warranted. To summarize briefly, no evidence was obtained for a therapeutic action of catalase on a rat or on a mouse tumor.

Experimental

Catalase was supplied by Puig Muset, Scientific Director of the P.E.V.Y.A. laboratories, Barcelona, Spain. These laboratories produce a dry, stable, soluble, extremely active catalase preparation designated Caperase. This product dissolves readily in minimal amounts of saline to produce a solution of many times the activity of the most potent American products.

Mice were CF No. 1 females, initially weighing about 25 g, and the tumor employed was the K_2ACLD ascites tumor. Rats were Sprague-Dawley males, weighing about 125 g at the start of the experiment; the tumor used was the Walker carcinosarcoma.

The Caperase was dissolved in saline at such a level as to give the mice, in each dose, approximately 1.9 times their total body catalase, and the rats approximately 2.4 times their total body catalase. This was accomplished with intraperitoneal injections of 0.25 ml to the mice and 1.0 ml to the rats.

On day zero, groups of 10 mice were implanted intraperitoneally with 0.1 ml of ascites fluid, and groups of 10 rats were implanted subcutaneously with 0.1 ml of minced Walker tumor. One group of mice received Caperase on days 1, 2, and 3; one group received Caperase on days 1, 3, and 5; and one group received no Caperase. In addition, one group of normal (tumor-free) mice received Caperase on days 1, 2, and 3, and one group received Caperase on days 1, 3, and 5.

The rats were also tested in groups of 10. One group was untreated; one group received Caperase on days 1, 2, and 3; and one group was treated on days 1 and 3 only. Only one group of control (tumor-free) rats was used; these received Caperase on days 1, 2, and 3. All tumor-bearing rats were sacrificed on day 9 because the tumors had become relatively enormous - many had ulcerated through the skin, and it was evident that the animals could not survive much longer.

Results

There were no deaths among the normal mice receiving Caperase. Average survival time for the untreated mice was 12.7 days. For the mice treated on days 1, 2, and 3 it was 8.4 days (or, if one omits 4 early deaths, presumably due to the Caperase, 10.7 days). For the mice receiving Caperase on days 1, 3, and 5 it was 12.8 days. Some minor differences are detectable in the weight curves, but these are not to be considered significant, first because the Caperase doses were so near the toxic level, and second because of the variability one often sees in such ascites-bearing mice. The results of the rat experiment are summarized in Table 50. Catalase was therapeutically ineffective against both the mouse and the rat tumor.

TABLE 50

Effect of Caperase (catalase) on rats bearing the Walker carcinosarcoma

Group	A	B	C	D
Tumor implanted	Yes	Yes	Yes	No
Caperase injected on days	-	1,2,3	1,3	1,2,3
Average initial weight, g	134	136	139	138
Average terminal weight, g	195	188	196	190
Average weight of tumor, g	9.5	9.1	8.9	-
Tumor as % of body weight	4.9	4.7	4.5	-

References

1. Puig Muset, P. About the Possibility of a Demalignant Treatment of Cancer. (monograph) Barcelona, Spain, June 26, 1960.

2. Warburg, O., K. Gawehn, and A. W. Geissler. Über die Wirkung von Wasserstoffperoxyd auf Krebszellen und auf embryonale Zellen. Z. Naturforsch. 12b 393-395 (1957).

3. Warburg, O., W. Schröder, H. Gewitz, and W. Völker. Über die selektive Wirkung der Röntgenstrahlen auf Krebszellen. Naturwiss. 45 192-193 (1958).

4. Warburg, O., W. Schröder, H. S. Gewitz, and W. Völker. Manometrisches Röntgenstrahlen-Aktinometer und über die Wirkung der Röntgenstrahlen auf die Gärung von Krebszellen. Z. Naturforsch. 13b 591-596 (1958).

3-AMINO-1,2,4,-TRIAZOLE

X. The Effect of Chemically Modified Aminotriazole on Erythrocyte Catalase

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Holman's claim[1] of curing the rat Walker carcinosarcoma by incorporating hydrogen peroxide in the drinking water has been repeatedly refuted, the list by now being too long to incorporate in a progress report. However, Warburg et al.,[2] have presented a theoretical basis for the argument that if the tumor-bearing animal could somehow be rendered catalase-free, then endogenous or exogenous H_2O_2 (e.g., radiation-produced) might prove carcinolytic.

We have previously demonstrated[3] that 3-amino-1,2,4-triazole (AT), upon injection or dietary ingestion, will strongly inhibit the catalase of most solid tissues but not that of the erythrocyte. We have shown the reason for non-inhibition of the erythrocyte catalase to be inability of AT to penetrate into the erythrocyte.[4]

We have, therefore, been interested in AT derivatives that might have the twin properties of (a) penetrating the erythrocyte and (b) then inhibiting the catalase therein. To accomplish the latter, it may be necessary that AT derivatives be converted within the cell to free AT.

A recent paper by van den Bos[5] seemed to offer some hope in this direction. This author described the synthesis of several derivatives of AT. Among these the most readily synthesized compounds were x-acetyl-3-amino-1,2,4-triazole (xAcAT) and 3-acetylamino-1,2,4-triazole (3AcAT). The following report describes some preliminary experiments with these two compounds.

Methods

Catalase was assayed by the perborate technique.[6] AT and "AT-like" chromogens were analyzed as described earlier.[7] Mice were CF No. 1 females, and all injections were intraperitoneal. Because of their limited solubility (see below), the xAcAT and the 3AcAT were generally injected as aqueous suspensions.

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Results

Properties of xAcAT and 3AcAT

Solubility. Whereas AT itself is extremely soluble in water (20% aqueous solutions are readily prepared), the two acetylated derivatives are much less soluble. The xAcAT appears to dissolve to the extent of approximately 1.0% and the 3AcAT, approximately 0.2%. Both of these figures are subject to considerable error, because of slow hydrolysis in aqueous solution and probable conversion of xAcAT to 3AcAT in aqueous solution. The error is probably greater for xAcAT, because both these factors play important roles. In fact, it has been observed that saturated, filtered solutions of xAcAT deposit a precipitate after a period of time; this may indicate conversion of 3AcAT.

Chromogenic value. Our previously described[7] method for analysis of AT consists of diazotizing with nitrous acid, coupling with chromotropic acid, and heating at 100°C. When this test is applied to xAcAT, and 3AcAT accurate interpretation of the results is difficult because of possible conversion of either compound to AT, and possible conversion of xAcAT to 3AcAT.

However, the experiment was performed, using serial dilutions of the above "saturated solutions" and assuming concentrations as noted above. Expressed as AT color value, we find that 1 mg of xAcAT yields a color equivalent to 1.25 mg of AT, and 1 mg of 3AcAT yields a color equivalent to 0.03 mg of AT.

The latter figure is undoubtedly due to the fact that the 3AcAT no longer has a free amino group for diazotization. The high value for the xAcAT may indicate that the additional acetyl group confers augmented chromogenicity on the original AT, or it may simply reflect the uncertainty of the original measurement of solubility.

In vitro penetration of AT, xAcAT, and 3AcAT into the mouse erythrocyte

Chemical test. We have earlier demonstrated[4] that if mammalian erythrocytes are suspended in saline and incubated at 37°C in the presence of AT, the catalase of the red cells is not inhibited. If AT is injected, the washed blood cells contain approximately 12% of the AT found in plasma or in liver.

In the present test, mouse erythrocytes were suspended in saline and incubated at 37°C with AT, xAcAT, 3AcAT, or with no additional agent. After 30 min, the cells were washed and centrifuged three times with saline, then lysed with distilled water and analyzed for AT-like color as described above. Although in this preliminary experiment sample sizes for assay were unfortunately selected in an inaccurate color range, the results (Table 51) nevertheless show clear-cut distinctions.

TABLE 51

AT-like color from erythrocytes incubated in the presence of AT, xAcAT and 3AcAT

Added agent	AT-like color, μg/ml
None	4
AT	18
xAcAT	ca. 120
3AcAT	ca. 100

Since, as indicated earlier, the xAcAT yields over 40 times the AT-like color of 3AcAT, it would appear from these results that (a) AT penetrated the cells to a negligible extent; and (b) both the xAcAT and the 3AcAT penetrated considerably more, the 3AcAT by far more than the xAcAT. This last point, of course, is quite conjectural, due to the possibility of enzymatic hydrolysis within the cell.

Enzymatic test. A similar experiment was performed in which the lysed material, instead of being analyzed for AT-like color, was assayed for catalase activity. Again distinct effects were observed (Table 52) although these did not exactly parallel the chemical data. It thus appears that, despite the greater apparent penetration of the 3AcAT in vitro, as indicated by chemical analysis, the xAcAT was the more inhibitory of red cell catalase in vitro.

TABLE 52

Catalase activity of erythrocytes incubated in the presence of AT, xAcAT, and 3AcAT

Added agent	Catalase activity (perborate units per ml packed cells)	Inhibition, %
None	376	-
AT	366	3
xAcAT	156	59
3AcAT	352	6

In vivo effect of AT, xAcAT, and 3AcAT on mouse erythrocyte catalase

Single injection. Three control (saline-injected) and 3 experimental mice were individually assayed, and the average values are shown in Table 53. In each case, the agent was injected as a 1 M solution (or suspension, in the case of xAcAT and 3AcAT), at 0.01 ml/g body weight. The mice were sacrificed 60 min after the injection.

TABLE 53

In vivo effect of single injections of AT derivatives on mouse erythrocyte catalase

Experiment	Agent tested	Blood catalase (perborate units/ml)	Inhibition, %
1	Saline	186	-
	AT	191	0
	xAcAT	171	8
2	Saline	200	-
	3AcAT	200	0

Multiple injections. The agent was injected 3 times, at 30-min intervals, and the mice were sacrificed after another 30 min. Each dose was 0.01 ml/g body weight, with the concentration of the agent at 0.25 M.

It is clear (Table 54) that suggestive but not significant evidence has been obtained for an in vivo effect of these agents on erythrocyte catalase.

TABLE 54

In vivo effect of multiple injections of AT derivatives on mouse erythrocyte catalase

Experiment	Agent tested	Blood catalase (perborate units/ml)	Inhibition, %
1	Saline	230	-
	3AcAT	198	14
2	Saline	199	-
	xAcAT	185	7

Summary

Two chemicals, x-acetyl-3-amino-1,2,4-triazole (xAcAT) and 3-acetylamino-1,2,4-triazole (3AcAT) have been tested as potential inhibitors of the catalase activity of intact erythrocytes.

By analysis for 3-amino-1,2,4-triazole (AT)-like color, evidence has been obtained that both the xAcAT and the 3AcAT are capable of penetrating the intact erythrocyte in vitro.

By enzymatic assay, it has been found that xAcAT, but not 3AcAT, significantly reduces the catalase activity of intact erythrocytes in vitro.

No evidence has yet been obtained for significant effect of either agent on erythrocyte catalase after in vivo injection. It seems likely that at least some of the difficulty encountered is due to limited solubility and stability of these agents.

We wish to thank Dr. Norman Frigerio for the synthesis of xAcAT and 3AcAT and for helpful discussion regarding their chemistry.

References

1. Holman, R. A. A method of destroying a malignant rat tumor in vivo. Nature 179 1033 (1957).

2. Warburg, O., K. Gawehn, and A. Geissler. Über die Wirkung von Wasserstoffperoxyd auf Krebszellen und auf embryonale Zellen. Z. Naturforsch. 12b 393-395 (1957).

3. 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).

4. Feinstein, R. N., and J. Dainko. Use of 3-amino-1,2,4-triazole to reduce body catalase activity in cancer studies. Cancer Research 19 612-617 (1959).

5. van den Bos, B. G. Investigations on pesticidal phosphorus compounds. II. On the structure of phosphorus compounds derived from 3-amino-1,2,4-triazole. Recueil Trav. chim. Pays-Bas 79 836-842 (1960).

6. Feinstein, R. N. Perborate as substrate in a new assay of catalase. J. Biol. Chem. 180 1197-1202 (1949).

7. Green, F. O., and R. N. Feinstein. Quantitative estimation of 3-amino-1,2,4-triazole. Anal. Chem. 29 1658-1660 (1957).

THE CORRELATION OF AUTORADIOGRAPHIC GRAIN COUNTS AND TRITIUM CONCENTRATION IN TISSUE SECTIONS CONTAINING TRITIATED THYMIDINE

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Tritium-labeled thymidine has been extensively used in recent years to investigate the kinetics of cellular proliferation in a variety of normal and pathological tissues. It is known that thymidine is incorporated into the nuclei of cells synthesizing DNA prior to mitosis[1] but little quantitative information is available on its uptake by single cells. Also dependent upon quantitative data is the problem of radiation dosimetry from intranuclear tritium. This problem has more than theoretical significance, since it has been shown that tritiated thymidine can produce radiation damage and death in some of the labeled cells.[2,3] For these purposes, measurements of total tritium activity in tissue samples[4] are inadequate because of the nonuniform distribution of thymidine in living tissue and the short range of the tritium beta particle. Under these conditions, 90% of the energy of the disintegrations is dissipated within the nucleus,[5] and the customary calculation of radiation dose in terms of energy absorbed per unit mass of tissue is inadequate. In order to correlate dose with biological effect the distribution of dose on a microscopic scale must be considered.

Both of these problems can be solved by high-resolution autoradiography, in which the type and number of labeled cells and the number of silver grains above each labeled cell can be determined with reasonable accuracy. However, in order to evaluate the absolute concentration of an isotope by autoradiographic methods, it is first necessary to determine the number of disintegrations required, on the average, to activate one silver grain above a labeled locus. Such determinations are available for most of the commonly used radioisotopes,[6] in which the geometric efficiency is taken as 50% and the overall efficiency is determined by the grain yield, that is, the number of grains per incident electron.[7,8] In the case of tritium, knowledge of the grain yield is not sufficient for quantitative purposes because its geometric efficiency is unknown, although some estimates have been made.[5,9,10]

The experiment to be described is an attempt to establish a correlation between the number of developed silver grains in a stripping-film emulsion and the concentration of tritium in the labeled cells of an underlying tissue section.

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Materials and Methods

General plan of the experiment. CAF_1 female mice, 5 months old and with a mean body weight of 26.2 g, were randomized into 4 pairs, each of which received intraperitoneal injections of tritiated thymidine as shown in Table 55. The tritiated thymidine (Schwartz Laboratories, Mount Vernon, N.Y.) had a specific activity of 360 mc/mM, and all animals were sacrificed by cervical dislocation 24 hr after the first or the only injection. The lungs were removed and weighed, and one lung was frozen for studies with the liquid scintillation counter,[4] while the other was fixed in formalin and processed as described in the section on autoradiographic technique. After the autoradiographs had been examined, the amount of tritium in the same sections was determined by gas-phase counting.

TABLE 55

Uptake of tritiated thymidine by the lungs of CAF_1 female mice*

Group no.	Amount of H^3-thymidine injected, μc/g body wt	No. of injections	μc/mg wet weight of lung, 1 x 10^3
1	1.25	1	0.097
2	2.50	2 (12)**	0.126
3	3.75	3 (6)**	0.252
4	7.50	6 (4)**	0.435

* Two animals per group.

** Values in parentheses are hours between injections.

Autoradiographic technique. The stripping-film technique described by Pelc[11] was used. The lungs were fixed in 10% neutral buffered formalin (Lillie's formula),[12] washed for 24 hr in running water, dehydrated and embedded in paraffin. Sections cut at 3μ were mounted on clean glass slides, hydrated, and covered with strips of Kodak Ltd. AR. 10 Autoradiographic film. The film strips, 38 by 36 mm, were floated on distilled water at 25°C for 5 min prior to mounting. The slides were then dried in a stream of warm air for 10 min and exposed in light-tight plastic boxes containing a small amount of Drierite ®. The exposure time, in the refrigerator at 4°C, was 30 days. Developing, fixing and staining of the autoradiographs were carried out at a temperature of 18°C. The slides were developed for 5 min in Kodak D-19 Developer (Eastman Kodak), washed for 30 sec in distilled water, and fixed for 8 min in Kodak Acid Fixer (Eastman Kodak), diluted 1:1. After washing for 30 min in 3 changes of distilled water, the slides were stained for 15 min with Ehrlich Acid Hematoxylin (Lillie's

formula, Reference 12, p. 57), differentiated in 0.2% HCl, blued in 0.5% sodium acetate in distilled water, and counterstained with eosin. The excess film was trimmed away and the slides were air-dried and mounted with Permount.

Grain counting. The number of labeled cells and the number of grains were determined visually under oil immersion. As the background fog was of the order of 0.2 grains per 100 μ^2, it was relatively easy to identify labeled cells. A cell was considered labeled when it had 3 grains or more; more than 95% of the labeled cells, however, had 10 grains or more. In the animals of the first 3 groups, grain counts performed by two different observers were in good agreement. In the animals receiving 6 doses of tritiated thymidine, clumping of grains made the counts less accurate.

At first, the grains were tallied, one by one, for each section, but it was soon realized that the mean grain count per labeled cell, when performed on samples of 50 cells, was remarkably constant. It was decided then to count all labeled cells in each section, and to determine the mean grain count per labeled cell for each section by averaging the grain counts of 5 separate 50-cell samples taken at random. The total number of grains per section was then obtained by multiplying the total number of labeled cells by the mean grain count per labeled cell.

Gas-phase counting. The radioactive assay of tritium β-particles with a maximum energy of 18.9 kev was obtained by counting the tissue sections, in the gas phase, as part of the counter filling for a glass proportional-counting tube.[13] In this method the single tissue sections were combusted in a sealed tube over nickel oxide and an excess of zinc for 1 hr at 660 ± 10°C, yielding a mixture of hydrogen and methane.[14] After the combustion was completed, the sample was introduced into the counting tube by means of a Toepler pump; the last portion was swept in by the P-10 gas (90 argon - 10 methane) introduced to bring the total pressure in the counting tube to one atmosphere. With this system, a rate of 10 disintegrations per minute is detectable with a standard deviation of ± 10%. The counting efficiency within the cathode volume for the tritium samples was determined by using a National Bureau of Standards tritiated toluene standard, and the standard deviation of the results for a series of 10 runs of the same sample of tritiated toluene was ± 1.9%.

Results

Table 55 shows the total activity per mg of lung tissue, as measured in the liquid scintillation counter. The amount of tritium per unit weight of tissue increases almost linearly with increasing doses of thymidine. Table 56 shows the correlation between the number of activated silver grains in the stripping-film emulsion and the amount of tritium present within the volume

of the underlying tissue section, 3 μ thick. As the number of grains per section represents the total of a 30-day exposure, while the amount of tritium per section is expressed in disintegrations per minute (dpm), the autoradiographic efficiency is calculated through the formula:

$$\text{eff.} = \frac{\text{total grain count}/30}{\text{dpm} \times 24 \times 60} \times 100 \quad .$$

The over-all autoradiographic efficiency increases with increasing doses of thymidine, varying from about 3 per thousand at the lowest dose level to almost 1% at the highest dose level.

TABLE 56

Efficiency of autoradiographic stripping-film in sections of lungs labeled with H^3-thymidine

Group no.	Section no.	Total no. of grains per section (30-day exposure)	Amount of H^3- in section, dpm	Efficiency $\left[\frac{\text{grain count}/30}{(\text{dpm} \cdot 24 \cdot 60)}\right] \cdot 100$
1	20-4	19,018	197	0.250
1	20-6	21,625	137	0.366
2	22-4	23,556	121	0.444
2	22-6	26,450	116	0.529
3	24-2	29,994	127	0.550
3	25-6	36,860	131	0.652
4	26-2	58,908	179	0.765
4	27-4	84,557	158	1.24

Table 57 gives the number of labeled cells per section and the mean grain count per labeled cell in the various samples. The majority of the labeled cells were found in small collections of lymphocytes in perivascular and peribronchial tissues and among connective tissue cells in the alveolar septa, especially in the subpleural regions. Epithelial cells of the bronchi and endothelial cells were very rarely labeled.

If we assume that the grain counts of groups 2 and 3 are the most accurate, the over-all efficiency of the stripping-film autoradiograph would be in the order of 5 per thousand, that is each grain above a labeled cell stands for 200 disintegrations. In any case, since the values in the various groups are within an order of magnitude, it can be safely stated that, in a tissue section 3 μ thick, 200 disintegrations from tritium are required to yield from 1 to 2 grains in the emulsion overlying the labeled locus.

TABLE 57

Number of labeled cells and mean grain count in sections of lungs labeled with H^3-thymidine

Group no.	Section no.	No. of labeled cells per section	Mean grain count per labeled cell
1	20-4	625	30.4
1	20-6	701	30.9
2	22-4	587	40.1
2	22-6	657	40.3
3	24-2	632	47.5
3	25-6	791	46.5
4	26-2	919	64.1
4	27-4	1313	64.4

Discussion

The experimental error. The efficiency of an autoradiographic technique is affected by a number of factors, among which are the autoradiographic method used,[15,16] the type, energy and angle of incidence of the particles,[8] the speed of the emulsion,[6] the latent image fading,[17] the desensitizing action that a tissue section exerts over an emulsion[18] and the effect of staining.[19]

The meaning of autoradiographic efficiency. Apart from the previous considerations, any correlation between number of grains and number of tritium atoms in a labeled locus depends at least upon another variable, namely the thickness of the tissue section. As mentioned above, the autoradiographic efficiency described in this experiment indicates only the number of grains developable for a given amount of tritium activity in the section and does not reflect the true efficiency of the emulsion, that is the number of developed grains per incident β-particle. Even with more energetic emitters, only electrons emitted in the direction of the film can possibly be utilized, and a geometric efficiency of 50% can be taken as maximal.[5,7] With tritium the situation is further complicated by the fact that particles of average energy (5.7 kev) have a range of only about 1 μ (only a very few of the tritium electrons of energy greater than the average have a range greater than 3 μ): in tissue sections several microns thick, the precise depth of the radioactivity in the section will affect the percentage of β-particles that get through to the sensitive emulsion. This decreased geometric efficiency is illustrated graphically by the radiation isodose curves above a point source of tritium constructed by Robertson et al.:[18] the number of electrons reaching the emulsion decreases with increasing

distance and increasing deviation from zero axis. The efficiency of the autoradiographic procedure for tritium should vary inversely with the thickness of the tissue section.

For these same reasons, however, a 3-μ section can be considered for all practical purposes a section of infinite thickness; that is, although the autoradiographic efficiency will decrease with increasing thickness, for any section more than 3μ thick the number of grains in the stripping emulsion will remain constant if the isotope concentration also remains constant.

The concentration of tritium per labeled nucleus. The relevant figures for determining the amount of thymidine incorporated into a single cell and the radiation dosimetry are the mean grain count and the volume of the nucleus. The number of grains above an individual cell is determined only in part by the absolute concentration of tritium in the nucleus, and largely by the distance between the nucleus and the emulsion and by the area of the nuclear cross section nearest to the emulsion. In a given cell population, this results in a "normal" distribution of individual grain counts, where the mode coincides with the mean grain count of the population.[4] The mean grain count, then, reflects, on the average, the number of tritium atoms disintegrating in each cell nucleus.

If the values established in the present experiment are accepted, namely, that 200 disintegrations are required on the average to activate one silver grain above a labeled nucleus, then, to obtain a mean count of 30 grains per cell, a total of 6,000 disintegrations must occur at each locus. Since tritium decays at the rate of 0.5% per month, 122 x 10^6 tritium atoms must be present in each locus to provide a mean grain count of 30. This, however, is the number of tritium atoms that must be present in the volume of a cell nucleus with a thickness of 3μ: assuming the nucleus to be a sphere with a mean diameter of 7 μ,[7] this number must be multiplied by 2.3 to obtain the number of tritium atoms present in the nucleus of a cell with a mean grain count of 30. The calculated value would then be 2.8 x 10^6 tritium atoms per nucleus, and this value is applicable to any tissue section more than 3 μ in thickness in which the labeled cells have a mean grain count of 30. Our results differ by a factor of 8 from the value estimated by Lajtha and Oliver:[5] 3.6 x 10^5 atoms to produce 30 grains per cell in a section after 25 days exposure. These authors had assumed an over-all efficiency of 2.5% for a section and 5% for a smear, in agreement with the efficiency calculated by Hughes,[9] of 1 grain per 20 disintegrations.

Once the average number of tritium atoms per nucleus can be established from a mean grain count, the calculations relating to the amount of thymidine incorporated and the radiation dose delivered can easily be performed. The number of tritium atoms can be translated into microcuries, and, knowing the specific activity of the injected thymidine, into moles

incorporated. Similarly the radiation dose in a nucleus with a volume of 500 μ^3 can be calculated from the isodose curves constructed by Robertson et al.,[20] for tritium, or from the data of Lajtha and Oliver.[5]

References

1. Hughes, W. L., V. P. Bond, G. Brecher, R. 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. 44 476-483 (1958).

2. Johnson, H. A., and E. P. Cronkite. The effect of tritiated thymidine on mouse spermatogonia. Radiation Research 11 825-831 (1959).

3. Lisco, H., E. T. Nishimura, R. Baserga, and W. E. Kisieleski. The effect of tritiated thymidine on the growth of Ehrlich ascites tumor in vivo. Lab. Invest. 10 435-443 (1961).

4. Baserga, R., W. E. Kisieleski, and K. Halvorsen. A study on the establishment and growth of tumor metastases with tritiated thymidine. Cancer Research 20 910-917 (1960).

5. Lajtha, L. G., and R. Oliver. The application of autoradiography in the study of nucleic acid metabolism. Lab Invest. 8 214-221 (1959).

6. Herz, R. H. Photographic fundamentals of autoradiography. Nucleonics 9 24-39 (1951).

7. Doniach, I., and S. R. Pelc. Autoradiographic technique. Brit. J. Radiol. 23 184-192 (1950).

8. Norris, W. P., and L. A. Woodruff. The fundamentals of radioautography. Ann. Rev. Nuclear Sci. 5 297-326 (1955).

9. Hughes, W. L. Autoradiography with tritium. In Symposium on Tritium in Tracer Applications, Boston: New England Nuclear Corp., 1957. pp. 38-40.

10. Cronkite, E. P. Discussion of radiation effects of cellular labels. In The Kinetics of Cellular Proliferation, Ed. F. Stohlman. New York: Grune and Stratton, 1959. p. 137.

11. Pelc, E. R. The stripping-film technique of autoradiography. Intern. J. Appl. Radiation and Isotopes 1 172-177 (1956).

12. Lillie, R. D., Histopathologic Technic, Philadelphia: The Blakiston Co., 1948.

13. Bernstein, W., and R. Ballentine. Gas phase counting of low energy beta-emitters. Rev. Sci. Instr. 21 158 (1950).

14. Wilzbach, K. E., L. Kaplan, and W. G. Brown. The preparation of gas for assay of tritium in organic compounds. Science 118 522 (1953).

15 Pelc, S. R. On the question of automatic or visual grain counting. Lab. Invest. 8 127-129 (1959).

16. Messier, E., and C. P. Leblond. Preparation of coated radioautographs by dipping sections in fluid emulsion. Proc. Soc. Exptl. Biol. Med. 96 7-10 (1957).

17. Herz, R. H. Methods to improve the performance of stripping emulsions. Lab. Invest. 8 71-75 (1959).

18. Tonna, E. A., and E. P. Cronkite. Factors which influence the latent image in autoradiography. Stain Technol. 33 255-260 (1958).

19. Schooley, J. C., B. J. Bryant, and L. S. Kelly. Preliminary autoradiographic observations of cellular proliferation in lymphoid tissues, using tritiated thymidine. In The Kinetics of Cellular Proliferation, Ed. F. Stohlman. New York: Grune and Stratton, 1959 pp. 208-217.

20. Robertson, J. S., V. P. Bond, and E. P. Cronkite. Resolution and image spread in autoradiographs of tritium-labeled cells. Intern. J. Appl. Radiation and Isotopes 7 33-37 (1959).

DNA SYNTHESIS OF MAMMALIAN KIDNEY CELLS IN TISSUE CULTURE AFTER SINGLE AND PERIODIC DOSES OF IRRADIATION

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Introduction

Several studies have been made of the ability of cells in vitro to synthesize DNA after single doses of irradiation, but little is known about the DNA synthesis of cells after repeated doses. Some investigations have shown that DNA synthesis is inhibited following irradiation,[1-8] while others have shown an appreciable increase.[9-12] This study will show a marked difference in the ability of two mammalian kidney cell lines from unrelated species and of different chromosome number to utilize tritiated thymidine in the synthesis of DNA, both under normal conditions and following single or periodic exposures to X-irradiation. Thymidine uptake was measured in a near-tetraploid Rhesus monkey kidney-cell line and in a diploid pig kidney-cell line.

The data will show that under the experimental conditions of tissue culture an established monkey kidney cell line utilizes tritiated thymidine at a very low rate, that the rate of uptake is materially increased by irradiation, and that pig kidney cells behave in a very different manner.

Materials and Methods

The monkey kidney cell line (Melnick strain) has been maintained in our laboratory for over 2 years on 70% Morgan's 199 synthetic nutrient, 20% bacto-tryptosephosphate broth, and 10% calf serum. We have established that the cell stemline is sub-tetraploid with a range of 76 to 78 chromosomes. The pig kidney cell, a diploid line, has a stemline number of 37 chromosomes. It is maintained on 95% Morgan's 199 synthetic nutrient and 5% calf serum. The chromosome karyotype has remained constant during the 11 months in our laboratory.

The procedures for preparing cultures for the experiments described in this paper were as follows. Monolayers of cells from either strain growing in stock bottles were trypsinized (monkey) or versene-trypsinized (pig) for 15 min at 37°C with a 0.25% solution. An aliquot of the cell suspension was counted with the Coulter electronic cell counter according to the method of Harris[13] and Peacock,[14] to estimate the total number of cells. A nutrient solution containing a known number of cells was prepared in growth nutrient. The culture vessel used throughout the experiments was the Leighton tube into which 1 to 2 x 10^5 cells were transferred. Duplicate or triplicate samples were prepared for each type of observation on each experimental group. For studying the incorporation

of tritiated thymidine, Leighton tubes containing glass coverslips (10 x 35 mm) were used to facilitate autoradiography, and for estimating total cell number the same type of tube was used without a coverslip.

Irradiation of the cultures began 18 hr after subcultivation. A 250 kvp - 30 ma X-ray machine with a half-value layer of 0.9 mm Cu and a 0.25 mm Cu and 1.0 mm Al filter was employed. The tubes with cells and nutrient were placed 65 cm from the X-ray tube and arranged radially on a rotating disk. The dose rate in all cases was 100 r/min and the doses employed were 200, 500 and 1000 r.

In the case of the monkey kidney cells, where periodic radiation was used, cell cultures were permitted to recover from the first, second, or third X-ray dose; they were then pooled one week after radiation and transferred to individual stock bottles until repetition of the irradiation 3 weeks later. Three main experimental groups are designated as follows: Group I consisted of cells given a single dose of irradiation. Group II consisted of cells from group I given a second dose of radiation. Group III were cells established from group II which were given a third dose of X-ray. Group IA represented a second single-dose experiment using cells of an unrelated subculture but from the same stemline. The same culturing procedures were followed for the unirradiated control cells.

The tritiated thymidine solution (Schwartz) used throughout the experiment was 1.0 mc/ml solution with a specific activity of 0.36 c/mM. It was prepared in Morgan's 199 nutrient to the desired concentration. At the specified interval of time after irradiation, 0.5 μc of tritiated thymidine was added to each of 2 or 3 culture tubes of each experimental group. These tubes were then incubated for 20 or 30 min only, at 37°C, after which the thymidine solution was removed and the coverslips with cells attached were washed with Tyrode's solution, fixed in Carnoy's fluid, and stored in 70% alcohol until the end of the experiment when all the coverslips were stained with Feulgen. Immediately following staining, Kodak AR-10 stripping film was applied to each coverslip, which had been previously mounted to a glass slide. After 14 days, the films were developed, washed, air-dried and mounted on glass slides for microscopic observation.

The number of cells with labeled nuclei above the background of 4 grains was determined from 2 or 3 samples which included a total of 500 to 2000 nuclei. From these same samples the number of mitotic figures was recorded and classified according to stages of mitosis. A few grain counts were made on representatively labeled cells in each case.

An estimation of the total cell population was made on unirradiated and irradiated cells growing in Leighton tubes with the Coulter Counter. Individual cells are enumerated by this method after versene trypsinization.

The preparation of chromosomes for karyotype analysis was by the smear or air-dry method of Hsu.(15) A chromosome analysis was made on cells 2 days after they had been subcultured to insure a high mitotic index, but a month after irradiation. Chromosome identification was made on metaphase spreads.

Results

Monkey Kidney Cells

The extent of the incorporation of tritiated thymidine by nuclei of control and irradiated monkey kidney cells during a period of 8 days after subcultivation and 6 days after 0 r, 500 r and 1000 r is shown in terms of the mean percentages of cells labeled (Table 58). The data for the normal monkey kidney cells show an initial high rate of incorporation (7-19%) of tritiated thymidine 18 to 24 hr after subcultivation (with the exception of group III), followed by a sharp decrease by 42 hr and a leveling at about 4% thereafter. The peak of incorporation at 24 hr could reflect a synchronous cell population appearing 8 to 10 hr after trypsination and subcultivation. To examine this, hourly determinations of the mitotic indices were made for 10 hr after subcultivation. It was found that within 2 hr the mitotic index dropped from 3.5% to 0.5% or less, and all cells in mitosis eventually disappeared. About 10 hr after subcultivation there was a marked increase in mitotic figures, indicating that the cells were in a phase of synchrony which lasted for about 24 hr. After the initial uptake in all 4 control groups, the cells incorporated tritiated thymidine at a surprisingly low rate as compared to the control pig kidney cells.

TABLE 58

Mean percentage of monkey kidney cells labeled by tritiated thymidine and percentage of cells in mitosis at various times after irradiation

Hours after irradiation	Group I						Group II				Group III				Group IA			
	Percent labeled			Percent mitosis			Percent labeled		Percent mitosis		Percent labeled		Percent mitosis		Percent labeled		Percent mitosis	
	Control	500 r	1000 r	Control	500 r	1000 r	Control	500 r	Control	500 r	Control	500 r	Control	500 r	Control	500 r	Control	500 r
0	70	70	70	25	25	25	70	70	30	30	07	07	07	07	-	-	15	15
6	192	83	158	30	08	05	74	128	25	<01	10	14	10	14	114	26	14	05
24	27	93	05	20	53	15	11	110	13	40	120	133	25	33	43	175	37	43
48	28	162	06	20	31	71	09	158	12	29	10	152	15	10	28	191	21	17
72	-	-	-		-	-	05	83	10	28	36	266	29	09	28	26	07	22
144	26	03	<01	18	30	<01			-	-	01	08	12	05	92	03	23	04

Irradiation of the monkey kidney cells increased the number of cells incorporating tritiated thymidine. After a single or repeated dose of 500 r to the cells, uptake by groups IA and III decreased at 6 hr after irradiation; this was not seen in groups I and II. In all 4 irradiated groups incorporation was markedly increased at 24 hr, and utilization of tritiated thymidine continued at a high level. This high incorporation rate relative to that of the controls persisted for 48 hr after irradiation but fell to control values or less by 72 and 144 hr after irradiation. The delay in the

increase in cells incorporating tritiated thymidine in group III (both controls and experimentals) is not understood, but it is noted that mitosis in this group is also retarded, suggesting a metabolic delay, possibly due to treatment with trypsin, or to other physicochemical disturbances.

The mean mitotic index for all four control groups during the 144-hr experiment averaged 2%; however, after repeated doses of 500 r a sharp decrease to 1% or less was followed by a marked increase of mitotic cells 24 and 48 hr after irradiation. This increase represents cells that were delayed in mitosis, and includes a preponderance of abnormal mitotic figures. It is noted that the mitotic index decreases 48 hr after irradiation and that the rate is dependent on the number of doses given.

Pig Kidney Cells

The incorporation of tritiated thymidine by the pig kidney cells is shown in Table 59, as the mean percentage of labeled cells after subcultivation and irradiation. Unlike the monkey kidney cells, the normal pig kidney cells incorporated tritiated thymidine at a high level (35%) over a 6-day period. The initial high uptake of thymidine in both the controls and the irradiated cells was similar to that observed in the monkey kidney cells. After exposure of the cells to a single dose of 200 r, 500 r, or 1000 r, the incorporation of thymidine decreased to 30% of the control values by 24 hr in all irradiated groups and did not return to control values even by the 6th day after irradiation.

TABLE 59

Mean percentage of pig kidney cells labeled and percentage of cells in mitosis after exposure to tritiated thymidine at various times after irradiation

Hours after irradiation	Percent labeled				Percent mitosis			
	Control	200 r	500 r	1000 r	Control	200 r	500 r	1000 r
(Subcultivation)*	32.8	32.8	32.8	32.8	4.9	4.9	4.9	4.9
0	43.6	43.6	43.6	43.6	3.0	3.0	3.0	3.0
6	32.1	40.9	39.2	31.1	2.9	0.6	0.2	<0.1
24	28.6	11.6	8.0	9.5	1.8	4.8	8.6	3.6
48	23.0	17.7	19.5	12.4	1.2	1.0	4.2	6.6
72	41.0	20.0	2.5	0.1	2.0	1.4	4.2	<0.1

*Time of transplantation, 18 hr before irradiation.

The mean percentage of pig kidney cells in mitosis after X-irradiation is shown in Table 59. An average of 2% of the cells were in mitosis during the 6-day period. As in the monkey kidney cells, irradiation of pig kidney cells resulted in a marked decrease in mitosis to less than 1% in all 3 groups at 6 hr after irradiation, followed by a sharp increase noted at 24 hr. The maximum increase after 500 r was seen 24 hr after irradiation, and after 1000 r, at 48 hr; 72 hr after 1000 r, no mitoses were seen. The mitotic cells were preponderantly abnormal.

Population Studies

Population growth data for normal and irradiated pig kidney cells are shown in Table 60. The initial inoculum for each tube was 6×10^3 cells. At the time of irradiation, two days after subcultivation, the cell population had increased to 2.4×10^4 cells as determined by the Coulter Counter.[13,14] The control cells exhibited a nearly exponential increase over a 6-day period with a doubling time of 1.15 days (27.6 hr) and then reached an asymptote. After 200 r there was a slight delay in cell growth during the second day, after which the growth rate was parallel to that of the controls. After 500 r the population decreased gradually beginning 2 days after irradiation, but by the 6th day the cell population was on an increase; during this period inhibition of mitosis appears to play a major role and cell death a minor one. Following 1000 r a marked decrease in the growth curve began at 2 days after irradiation and continued without recovery.

TABLE 60

Total number of cells in pig kidney cultures following irradiation (mean of 3 cultures)

Days after X-ray	Cells x 10^5			
	0 r	200 r	500 r	1000 r
(Subcultivation)*	0.06	0.06	0.06	0.06
0	0.24	0.24	0.24	0.24
1	0.60	0.60	0.61	0.66
2	1.10	0.87	0.76	0.59
3	1.92	1.45	0.70	0.47
4	3.99	2.22	0.54	0.18
6	7.26	6.64	1.72	0.25
12	16.50	15.70	6.22	0.23
14	17.60	14.30	7.64	0.17

* 18 hr before irradiation

Grain Counts

Approximate grain counts were made, under identical conditions of exposure, in representative cells in most of the experiments. These indicate the rate at which the labeled cells are taking up thymidine in synthesis of DNA. Such counts are necessary in order to detect differences in rate of uptake since a prolonged synthetic phase would increase the proportion of cells labeled, but this would occur concomitantly with a decrease in average grain count at a given synthetic rate.

Unirradiated pig kidney cells showed, at the 6-hr period, average grain counts of about 50; at 24 hr, about 100; and at 48 hr, about 35. These counts are substantially higher than any made on the monkey kidney, which, if conditions were identical, might have been expected to have higher grain counts per cell due to higher DNA content. Unirradiated monkey kidney cells showed counts between 10 and 20 at all time intervals; after 100 r, about the same; after 500 r, 20 to 30 at 24 and 48 hr and 12 to 15 at 6 hr; and after 100 r, 15 at 6 and 24 hr. It seems clear from this that grain counts ran parallel to percentages of labeled cells, while if the percentage had reflected different rates of synthesis alone, the grain counts would have been inversely related to percentages of labeled cells.

Chromosome Karyotypes

Monkey kidney cells that had recovered from three repeated doses of 500 r were cloned one month after the third dose of radiation. A few of the clones were isolated for study of their chromosome karyotypes, taken at metaphase, by the chromosome smear technique.[15] Among the abnormalities were chromatid and chromosome breaks, deletions, chromatid exchanges, chromosome exchanges and minute fragments. A chromosome marker with chromatids from a non-homologous chromosome was seen in many of the cells from the same clone, indicating persistence and duplication of this type of abnormality through many cell divisions. The time at which this and other abnormalities arose is not known since the cells were studied only at one month after the third dose of irradiation.

Pig kidney cells were cloned one month after a single dose of 500 r. Chromatid and chromosome breaks, inversions, deletions, iso-chromatid exchanges, chromatid exchanges, chromosome exchanges and other abnormalities were seen. Radiation damage appeared to be more severe after one single dose of 500 r than that seen in the monkey kidney cells after 3 doses of 500 r given 1 month apart. In one pig kidney cell as many as two single chromatid breaks, chromatid exchanges, and deletions were observed. Such abnormalities were most often seen in cells which had become polyploid. The occurrence of a chromosome with chromatids from a non-homologous chromosome similar to that seen in the monkey kidney cells was persistent in diploid cells and was isolated from one of

the clones whose mitotic cells carried the variant. This type of marker was described by Ruddle[16] in the same cell line after sublethal doses of irradiation The appearance of anaphase bridges with loss of chromosome fragments at anaphase was a common occurrence. Many abnormal metaphase structures appeared as well.

Discussion

The difference in the ability of two types of mammalian kidney cell growing in vitro to incorporate tritiated thymidine under the conditions of these experiments might be explained on the basis of a biochemical difference. Since the normal monkey kidney cells utilize thymidine at a very low rate during DNA synthesis, it is apparent that they use a different metabolic pathway for DNA synthesis than the normal pig kidney cells. It is possible that the monkey kidney cells make thymidine de novo for DNA synthesis. The fact that after three doses of 500 r they incorporated tritiated thymidine at a high rate compared to their controls, indicates that the irradiated cells are using exogenous thymidine for DNA synthesis. If this is so there must be a blocking of some key enzyme system necessary for de novo synthesis of DNA after irradiation. It is possible that the initial increase in thymidine incorporation by the normal monkey kidney cells may be dependent on a shifting of the intermediary pathway for de novo synthesis of DNA, due to subcultivation and synchrony of the cells, while later, the cells may show a decrease in uptake due both to a reversion to de novo DNA synthesis and to loss of the synchrony Painter and Robertson[10] reported an increase above controls in the labeling of HeLa S3 cells during the first few hours after irradiation, followed by a fall in the percentage of labeled cells below normal 24 hr after irradiation.

Experiments were conducted to determine whether the concentration of tritiated thymidine (0.5 μc/ml), the specific activity of 0.36 c/mM, or the length of time the tritiated thymidine solution remained on the cells. were sufficient to label normal monkey kidney cell nuclei properly. Exposure of the cells to 1 μc/ml (3.6 c/mM) in Morgan's 199 nutrient for $1\frac{1}{2}$ hr (10 times that used originally) resulted in no change in the uptake of the label Likewise, if the labeled thymidine were diluted in the complete basic nutrient or in Eagle's nutrient which contains no thymine, there was no difference in labeling. To ensure that removal of the unlabeled nutrient and addition of fresh nutrient with label did not alter the uptake of the label, concentrated or partially concentrated tritiated thymidine (0.5 μc/ml) was added directly to the unlabeled nutrient; no change in incorporation was observed.

In contrast to the monkey kidney cells the normal pig kidney cells incorporated labeled thymidine at a level of 30 to 40%, which would be expected at the concentrations and specific activity employed. However, the irradiated cells showed a high rate of thymidine incorporation only

initially, a fact that could also be explained on the basis of cells in synchrony after subcultivation. The marked reduction of mitosis 6 hr after irradiation was not paralleled by a reduction in the number of cells labeled at this time, but at 24 hr after irradiation, the number of cells labeled in any irradiation group fell to one-third of the controls. Mitotic figures increased markedly at 24 hr after irradiation, as a result of delay of cells in mitosis due to radiation damage.[2,17-19] There was no reduction in the growth curve 24 hr after irradiation; the doubling time (18 hr) for all the irradiated cells was the same as for the controls during this time, indicating that the cells were able to go through one division, regardless of the stage in the mitotic cycle in which they were irradiated. Injury to the mitotic apparatus of the dividing cells is manifested 48 hr after irradiation in the growth curves, which decline in all three irradiated groups.

Cells recovering from the highest dose of irradiation were subcultured for further study. After a single dose of 1000 r to the monkey kidney cells the incorporation rate of the label was high, as it was after 500 r, but after a second dose given one month later there was only a transient increase in the percentage of labeling with a decrease by 24 hr. The number of cells was few and 2 or 3 weeks later there were no survivors. In the case of the pig kidney cells we were unable to give a second dose of 1000 r as there were no survivors 4 weeks after irradiation. Also, it is occasionally impossible to carry survivors through a second subcultivation and irradiation after a single dose of 500 r. It appears that the pig kidney cells are more radiosensitive than the monkey kidney cells although the decrease in cell number in cultures that have resumed growth after a few days suggests that the LD_{50} is about the same for the two cell types. The greater sensitivity of the pig cells is supported by the fact that they exhibit a larger number of chromatid breaks, deletions, isochromatid exchanges, etc., at one month after a single dose of 500 r. Fewer of these abnormal mitotic figures are observed even after 3 doses of 500 r to the monkey kidney cells. Whether this may be due in part to recovery, by elimination of cells damaged by the first irradiation, remains to be verified. It seems possible that this process might be facilitated in the monkey cells, which have a larger complement of chromosomes.

It is conceivable that variations in the proportion of labeled cells might indicate differences in the rate of DNA synthesis, in which case a higher percentage of labeled cells might indicate a slower buildup of DNA during the synthetic phase, which was thereby prolonged. This seems to be negated by the fact that grain counts ran parallel to the proportion of cells labeled, being higher in the case of the pig kidney cells, lower in the unirradiated monkey kidney cells, and higher in the latter after irradiation.

The product of the grain counts and the proportion of labeled cells, gives the rate of average synthesis of DNA per cell in the cultures; it ranged from 7.4 to 27.6 in the unirradiated pig cells; 2.3 at 6 hr and

0.51 or less at 24 hr and later in unirradiated monkey cells; and 2.6 to 5.3 at the 24- and 48-hr periods after irradiation of these cells at 500 r.

One possible difference between diploid and tetraploid cells should be mentioned. Hauschka et al. [20] have shown that a tetraploid ascites tumor grows, in its later stages, at about one-half the rate of the corresponding diploid since the growth is limited by the mass of tumor rather than by the cell number, and that the duration of mitosis is prolonged as well as the duration of the intermitotic period; while this may also be true under the conditions of tissue culture, it can hardly account for the decreased rate of synthesis, both by individual cells and on the basis of the number of cells synthesizing DNA at a given time.

For normal growth, the monkey kidney has a requirement that the pig kidney does not, which is supplied by the addition of bacto-tryptose phosphate to its growth medium. The possibility that this material may either be protective against radiation or that it may be required for recovery of irradiated cells might explain the difference in response of the two types of cells, and this is now being investigated.

Summary

The effect of irradiation upon the uptake of tritiated thymidine by cells in vitro has been investigated, employing a pig kidney cell and a monkey kidney cell strain.

The two strains exhibit marked differences in thymidine uptake both in the unirradiated state and after irradiation. Thirty to forty percent of the pig cells incorporate thymidine and this rate of incorporation continues for several days after subcultivation; after irradiation, the extent of thymidine incorporation is reduced in relation to the X-ray dose (between 200 and 1000 r). Only a small percentage of monkey cells incorporate thymidine except for a period in the first day of subcultivation which may represent a period of synchrony or of recovery from injury of handling; on the other hand, these cells are proliferating at about the same rate as the pig cells. When the monkey cells are irradiated, they show a sharp increase in the percentage of labeling, which continues for several days. It is suggested that normal DNA synthesis in the monkey cells involves some alternate pathway of DNA synthesis not utilizing exogenous thymidine, but a de novo synthesis of DNA, and that a radiation-induced block of that pathway results in utilization of the labeled thymidine by the cells.

The pig cells are diploid and the monkey cells are sub-tetraploid. The pig cells are more radiosensitive, perhaps on this account; cultures of these cells fail to grow after a second irradiation with 500 r while the monkey cells grow well after three such irradiations. Alternative explanations of the differences in radiosensitivity are under consideration.

References

1. Lajtha, L. G., R. Oliver, T. Kumatori, and F. Ellis. On the mechanism of radiation effect on DNA synthesis. Radiation Research 8 1-16 (1958).

2. Harrington, H. Effect of irradiation on cell division and nucleic acid synthesis in strain U-12 fibroblasts. Biochim. Biophys. Acta 41 461-469 (1960).

3. Ord, M. G., and L. A. Stocken. The effect of X-radiation on rat thymus nucleic acids at short intervals after exposure in vivo. In Advances in Radiobiology, Eds. G. de Hevesy, A. Forssberg and J. Abbatt. Edinburgh: Oliver and Boyd, Ltd., 1957. P. 65.

4. Holmes, B. E. Influence of radiation on metabolism or regenerating rat liver. Ciba Foundation Symposium, Ionizing Radiations and Cell Metabolism. London: Churchill, 1956. pp. 225-226

5. Lesher, S., A. N. Stroud, and A. M. Brues. The effects of chronic irradiation on DNA synthesis in regenerating mouse liver. Cancer Research 20 1341-1346 (1960).

6. Pelc, S. R., and A. Howard. Effects of various doses of X-rays on the number of cells synthesizing deoxyribonucleic acid. Radiation Research 3 135-142 (1955).

7. Kelly, L. S. Effect of radiation on DNA synthesis in mammalian cells. Progress in Biophysics, Vol. 8. New York: Pergamon Press, 1957. pp. 143-163.

8. Looney, W. B., R. C. Campbell, and B. E. Holmes. The effect of irradiation on the replication of desoxyribonucleic acid in hepatocytes. Proc. Natl. Acad. Sci. 46 698-708 (1960).

9. Painter, R. B. Nucleic acid metabolism in HeLa S3 cells after X-ray induced mitotic delay. Radiation Research 13 726-736 (1960).

10. Painter, R. B., and J. S. Robertson. Effect of irradiation and theory of role of mitotic delay on the time course of labeling of HeLa S3 cells with tritiated thymidine. Radiation Research 11 206-217 (1959).

11. Stroud, A. N., A. M. Brues, and B. R. Svoboda. DNA and protein content of Krebs-2 mouse ascites tumor cells grown under heterologous conditions. Proc. Am. Assoc. Cancer Research 3 67 (1959).

12. Whitfield, J. F., and R. H. Rixon. Effects of X-radiation on multiplication and nucleic acid synthesis in cultures of L-strain mouse cells. Exptl. Cell Research 18 126-137 (1959).

13. Harris, M. Growth Measurements on monolayer cultures with an electronic cell counter. Cancer Research 19 1020-1024 (1959).

14. Peacock, A. C., G. Z. Williams, and H. F. Mengoli. Rapid electronic measurement of cell volume and distribution. J. Natl. Cancer Inst. 25 63-74 (1960).

15. Hsu, T. C. Personal communication.

16. Ruddle, F. H. Chromosome variation in clonal lines of tissue culture cells derived from pig kidney. Radiation Research 12 468 (1960).

17. Stroud, A. N., and A. M. Brues. Radiation effects in tissue culture. Texas Repts. Biol. Med. 12 931-944 (1954).

18. Stroud, A. N. The effects of continuous irradiation by tritium on cells cultivated in vitro. Ann. N. Y. Acad. Sci. 67 11-34 (1956).

19. Pomerat, C. M., M. V. Fernandes, Y. H. Nakanishi, and S. P. Kent. Irradiation of cells in tissue culture. V. The effect of gamma irradiation from a cobalt-60 source on human amnion cells in vitro. Z. Zellforsch. 48 1-9 (1958).

20. Hauschka, T. S., S. T. Grinell, L. Revesz and G. Klein. Quantitative studies on the multiplication of neoplastic cells in vitro. IV. Influence of doubled chromosome number on growth rate and final population size. J. Natl. Cancer Inst. 19 13-32 (1957).

PUBLICATIONS

January 1, 1960 through December 31, 1960

Ainsworth, E. J., and P. D. Forbes. Decreased Radiation Mortality in Mice Treated with a Pseudomonas Pyrogen. Radiation Research 12 417 (1960). Abstract.

Auerbach, H. The Problems of Testing Changes in Low Frequency Events in Human Populations. Oklahoma Conference - Radioisotopes in Agriculture, April 2-3, 1959, Stillwater, Oklahoma. USAEC Report TID-7578, pp. 23-26.

Barton, A. D. Soluble Proteins of the Cell Nucleus. The Cell Nucleus. London: Butterworth and Co. 1960. pp. 142-143.

Baserga, R., and W. E. Kisieleski. Increased Incidence of Tumor Metastases in Female Mice. Science 132 956-957 (1960).

Beams, H. W., R. L. King, T. N. Tahmisian, and R. Devine. Electron Microscopic Studies on Lophomonas striata with Special Reference to the Nature and Position of the Striations. J. Protozool. 7 91-101 (1960).

Beams, H. W., T. N. Tahmisian, E. Anderson, and R. Devine. Studies on The Fine Structure of Ultracentrifuged Spinal Ganglion Cells. Anat. Record 137 338 (1960). Abstract

Beams, H. W., T. N. Tahmisian, E. Anderson, and B. Wright. Electron Microscope Studies on the Nucleus and Associated Perinuclear Tubules of Lophomonas blattarum. Anat. Record 136 161 (1960). Abstract.

Beams, H. W., T. N. Tahmisian, R. L. Devine, and E. Anderson. An Electron Microscope Study on the Pharyngeal Glands of the Honeybee. J. Ultrastructure Research 3 155-170 (1959).

Brues, A. M. Critique of Mutational Theories of Carcinogenesis. Acta Intern. Union Contra Cancrum 16 415-417 (1960).

Brues, A. M. Nuclear Technology and Medicine in 1985. What's New (Abbott Laboratories) No. 220, pp. 28-29 (1960).

Brues, A. M. Prospects for a Systemic Therapy of Cancer. Symp. on Normal and Abnormal Differentiation and Development. Natl. Cancer Inst. Monograph No. 2, 1960. pp. 179-187.

PUBLICATIONS (Cont'd.)

Brues, A. M. Radiation Sickness. Medical Physics, ed. Otto Glasser. Chicago: The Yearbook Publishers, Inc., 1960, pp. 507-508.

Brues, A. M. Selected Materials on Radiation Protection Criteria and Standards: Their Basis and Use. Joint Committee on Atomic Energy, 86th Congress of the United States, Washington, D. C.: U. S. Government Printing Office, 1960. pp. 408-436.

Brues, A. M. Toxicity of Radioactive Isotopes. Medical Physics, ed. Otto Glasser. Chicago: The Yearbook Publishers, Inc., 1960. pp. 351-353.

Brues, A. M., H. Auerbach, and L. D. Marinelli. Letter to the Editor. Science 131 230 (1960).

Chorney, W., N. J. Scully, H. L. Crespi, and J. J. Katz. The Growth of Algae in Deuterium Oxide. Biochim. Biophys. Acta 37 280-287 (1960).

Chorney, W., N. J. Scully, C. H. Yang, H. D. Braymer, E. L. Murphy, and S. H. Wender. Methyl Esters of Quercetin in Tobacco. J. Organ. Chem. 25 2063-2064 (1960). Note.

Czajka, D. M., and A. J. Finkel. Effect of Deuterium Oxide on the Reproductive Potential of Mice. Ann. N. Y. Acad. Sci. 84 770-779 (1960).

Daniels, E. W. Survival and Cell Division of Amoebae Following the Microinjection of Homologous and Other Protoplasm. Anat. Record 137 348 (1960). Abstract.

Draper, L. R. The Hemolysin Response in Rabbits Immunized during Low-Level Cobalt-60 Gamma Irradiation. J. Infectious Diseases 107 34-42 (1960).

Ehret, C. F. Biological Organization. [Reply to Comments on Paper Published in Science 132 115 (1960)]. Science 132 1412 (1960). Letter.

Ehret, C. F. Organelle Systems and Biological Organization. Science 132 115-123 and cover (1960).

Ehret, C. F. Radiobiology. American People's Encyclopedia Yearbook. Chicago: Spencer Press, pp. 846-847 (1960).

PUBLICATIONS (Cont'd.)

Ehret, C. F. The Induction of Phase-Shift in Cellular Rhythmicity by Far Ultraviolet and Its Restoration by Visible Radiant Energy. Photoperiodism and Related Phenomena in Plants and Animals. AAAS Symposium, Washington, D. C., 1959, pp. 541-550.

Ehret, C. F. The Photobiology and Biochemistry of Circadian Rhythms in Nonphotosynthesizing Cells. Fed. Proc. 18 1232-1240 (1959).

Ehret, C. F., B. Smaller, E. L. Powers, and R. B. Webb. Thermal Annealment and Nitric Oxide Effects on Free Radicals in X-Irradiated Cells. Science 132 1768-1769 (1960)

Feinstein, R. N. Activation of the Neutral Deoxyribonuclease (DNAse I). J. Biol. Chem. 235 733-738 (1960).

Feinstein, R. N. Attempts at Protection of Mice Against Ionizing Radiation by Exogenous Catalase. Atompraxis 6 205-207 (1960).

Finkel, M. P. Life Shortening and Production of Tumors by Strontium-90. Science 132 1683-1684 (1960). Letter.

Finkel, M. P. Radiation: The Role of the Atomic Energy Commission. Health (Wisc. State Board of Health Bull.) 14 31-32 (1960).

Finkel, M. P., P. J. Bergstrand, and B. O. Biskis. Latency and Growth of Osteogenic Sarcomas Induced by Radiostrontium. Proc. Am. Assoc. Cancer Research 3 110 (1960). Abstract.

Finkel, M. P., P. J. Bergstrand, and B. O. Biskis. Latency and Growth of Osteogenic Sarcomas Induced by Radiostrontium: A Summary. Radiation Damage in Bone. Report of a Conf. on Relation of Radiation Damage to Radiation Dose in Bone, Oxford, April 10-14, 1960. Intern. Atomic Energy Agency, Vienna, 1960. STI-Pub/27, pp. 38-40. Abstract.

Finkel, M. P., P. J. Bergstrand, and B. O. Biskis. The Consequences of the Continuous Ingestion of Sr^{90} by Mice. Radiology 74 458-467 (1960).

Finkel, M. P., P. J. Bergstrand, and D. J. Graubard. The Uptake and Retention of Dietary Strontium-90. Radiation Research 12 434-435 (1960). Abstract.

PUBLICATIONS (Cont'd.)

Finkel, M. P., and B. O. Biskis. Radium Damage in Mouse Bone. Radiation Damage in Bone. Report of a Conf. on Relation of Radiation Damage to Radiation Dose in Bone, Oxford, April 10-14, 1960. Intern. Atomic Energy Agency, Vienna, 1960. STI-PUB/27, pp. 27-28. Abstract.

Finkel, M. P., B. O. Biskis, and P. J. Bergstrand. Radioisotope Toxicity: Significance of Chronic Administration. Symp. on Radioisotopes in the Biosphere, eds. R. S. Caldecott and L. A. Snyder. Minneapolis: Univ. Minn. Press, 1960. pp. 461-473.

Finkel, A. J., and D. M. Czajka. Effect of Deuterium Oxide on Ascites Tumor Growth in Mice. Ann. N. Y. Acad. Sci. 84 755-762 (1960).

Flynn, R. J. Notoedres muris Infestation of Rats. Proc. Animal Care Panel 10 69-70 (1960).

Fry, R. J., K. Hamilton, and H. Lisco. An Unusual Spontaneous Cardiac Lesion of Unknown Etiology in Mice. Fed. Proc. 19 109 (1960). Abstract.

Fry, R. J., S. Lesher, and H. I. Kohn. Renewal of Epithelial Cells in the Jejunum and Ileum of Mice of Three Age Groups. Radiation Research 12 435 (1960). Abstract.

Gatenby, J. B. Notes on the Gametogenesis of a Pulmonate Mollusc: An Electron Microscope Study. La Cellule 60 289-300 (1960).

Gatenby, J. B., and R. L. Devine. The Structure and Ageing of Sympathetic Neurons. J. Roy. Microscopical Soc. 79 1-12 (1960).

Gatenby, J. B., and T. N. Tahmisian. Centriole Adjunct, Centrioles, Mitochondria and Ergastoplasm in Orthopteran Spermatogenesis. La Cellule 60 105-134 (1959).

Gordon, S. A. Spectral Potentiation of X-Ray Induced Chromosomal Aberrations. Oklahoma Conference - Radioisotopes in Agriculture, April 2-3, 1959, Stillwater, Oklahoma. USAEC Report TID-7578, pp. 163-171.

Gordon, S. A., and K. Surrey. Red-Far-Red Interaction in Oxidative Phosphorylation. Radiation Research 12 325-339 (1960).

PUBLICATIONS (Cont'd.)

Grahn, D. Genetic Control of Physiological Processes: The Genetics of Radiation Toxicity in Animals. Symp. on Radioisotopes in the Biosphere, ed. R. S. Caldecott and L. A. Snyder. Minneapolis: Univ. Minn., 1960. pp. 181-200.

Grahn, D., and K. Hamilton. Genetics of Radiation Toxicity. Genetics Research, Division of Biology and Medicine. TID-4041 (1960), p. 6. Abstract.

Grieff, D., E. L. Powers, W. Kisieleski, and H. Pinkerton. The Effects of X-Rays and Beta Rays (Tritium) on the Growth of Rickettsia mooseri and Rickettsia akari in Embryonate Eggs. J. Exptl. Med. 3 841-849 (1960)

Gustafson, P. F. Assessment of the Radiation Dose Due to Fallout. Radiology 75 282-288 (1960).

Gustafson, P. F. Assessment of the Radiation Dose Due to Fallout. Selected Materials on Radiation Protection Criteria and Standards: Their Basis and Use. Joint Committee on Atomic Energy, 86th Congress of the United States. Washington, D. C.: U. S. Govt. Prtg. Office, 1960. pp. 797-816.

Gustafson, P. F., S. S. Brar, and M. A. Kerrigan. Variations of Fallout Radioactivity in Soil and Air. Radiation Research 12 440 (1960). Abstract

Jaroslow, B. N. Factors Associated with Initiation of the Immune Response. J. Infect. Dis. 107 56-64 (1960).

Jaroslow, B. N., and W. H. Taliaferro. Effects of X-Radiation on the Induction of Hemolysin Formation in Rabbits. Radiation Research 12 445 (1960). Abstract.

Kisieleski, W. E., R. Baserga, and K. Halvorsen. A Study on the Establishment and Growth of Tumor Metastases Using Tritiated Thymidine. Cancer Research 20 910-917 (1960).

Kisieleski, W. E., R. Baserga, and K. Halvorsen. A Study on the Establishment and Growth of Tumor Metastases with Tritiated Thymidine. Proc. Am. Assoc. Cancer Research 3 93 (1960). Abstract.

PUBLICATIONS (Cont'd.)

Klein, P. D. Dietary Fat and Plasma Cholesterol: A Speculative Review. Am. J. Clin. Nut. 8 104-111 (1960).

Kubitschek, H. E. "Classical" Mutation in the Chemostat. Biophys. Soc. Abstracts #II: 19 (1960).

Kubitschek, H. E. Delayed Phenotypic Expression of Mutation. Oklahoma Conf. - Radioisotopes in Agriculture, April 2-3, 1959. Stillwater, Oklahoma. USAEC Report TID-7578, pp. 173-180

Kubitschek, H. E. Electronic Measurement of Particle Size. Research 13 128-135 (1960).

Kubitschek, H. E. The Error Hypothesis of Mutation. Science 131 730 (1960).

Kubitschek, H. E., and H. E. Bendigkeit. The Mutation Process. Genetics Research, Division of Biology and Medicine. TID-4041 (1960), p. 7 Abstract.

Laird, A. K., and A. D. Barton. Cell Growth and the Development of Tumors. Proc. Am. Assoc. Cancer Research 3 34 (1959). Abstract.

Laird, A. K., and A. D. Barton. Cell Proliferation in Precancerous Liver: Linear Growth Curve. Nature 188 417-418 (1960). Letter.

Leone, C. A. Effects of γ-Rays on the Serologic Properties of Ovalbumin. I. Irradiated Lyophilized Protein. J. Immunol. 85 (2) 107-111 (1960).

Leone, C. A. Effects of γ-Rays on the Serologic Properties of Ovalbumin. II. Fractions from Irradiated Lyophilized Protein. J. Immunol. 85 (2) 112-119 (1960).

Leone, C. A. Effects of γ-Rays on the Serologic Properties of Ovalbumin. III. Irradiated Solutions. J. Immunol. 85 (3) 268-274 (1960).

Lesher, S., K. Hamilton, D. Grahn, and G. Sacher. The Causes of Death for LAF_1 Mice Exposed to Low Doses Daily, Co^{60}, Gamma Irradiation. Radiation Research 12 451 (1960). Abstract.

PUBLICATIONS (Cont'd.)

Lesher, S., A. N. Stroud, and A. M. Brues. The Effects of Chronic Irradiation on DNA Synthesis in the Regenerating Mouse Liver. Cancer Research 20 1341-1346 (1960).

Li, N. C., A. Lindenbaum, and J. M. White. Some Metal Complexes of Citric and Tricarballylic Acids. J. Inorg. Nucl. Chem. 12 122-128 (1959).

Lindenbaum, A., J. F. Fried, M. W. Rosenthal, and J. Schubert. Influence of Colloidal State on Plutonium Distribution and on Effectiveness of DTPA Therapy. Fed. Proc. 19 355 (1960). Abstract.

Lindenbaum, A., and J. Schubert. Sustained Action of Injected Chelating Agents. Nature 187 575-576 (1960). Letter

Lisco, H. Histopathologic Changes in Bones and Their Sequelae in Patients with Chronic Radium Poisoning. Radiation Damage in Bone. Report of a Conf. on Relation of Radiation Damage to Radiation Dose in Bone, Oxford, April 10-14, 1960. Intern. Atomic Energy Agency, Vienna, 1960. STI-PUB/27, pp. 21-23. Abstract.

Lisco, H., R. Baserga, and D. B. Cater. Delayed Effects of External Gamma Irradiation on the Bones of Rats. Radiation Damage in Bone. Report of a Conf. on Relation of Radiation Damage to Radiation Dose in Bone, Oxford, April 10-14, 1960. Intern. Atomic Energy Agency, Vienna, 1960. STI-PUB/27, p. 23. Abstract

Lisco, H., D. B. Cater, and R. Baserga. Radiographic Appearances of Bone Lesions in Rats Exposed to Local External Irradiation from Gamma Rays. Acta Radiol. 54 273-288 (1960).

Lisco, H., J. Vaughan, and L. F. Lamerton. The Relation of Radiation Dose in Bone. Radiation Damage in Bone. Report of a Conf. on Relation of Radiation Damage to Radiation Dose in Bone, Oxford, April 10-14, 1960. STI-PUB/27, pp. 7-20.

Longwell, A. C., and M. Mota. The Distribution of Cellular Matter During Meiosis. Endeavour 19 100-107 (1960).

Longwell, A. C., and G. Svihla. Specific Chromosomal Control of the Nucleolus and of the Cytoplasm in Wheat. Exptl. Cell Research 20 294-312 (1960).

PUBLICATIONS (Cont'd.)

Malone, T. E. Observations on the Histochemical Localization of Alkaline Glycerophosphatase in Developing Corpora Lutea of Albino Rats. Am. J. Anat. 106 41-43 (1960).

Maloney, M. A., and H. M. Patt. Radiation Effects on Neutrophil Balance. Ciba Foundation Symposium, 1960. pp. 262-270

Marshall, J. H., and M. P. Finkel. Comparison of Microdosimetry and Tumor Production for Ca^{45}, Sr^{90} and Ra^{226} in Mice. Radiation Damage in Bone. Report of a Conf. on Relation of Radiation Damage to Radiation Dose in Bone, Oxford, April 10-14, 1960. Intern. Atomic Energy, Vienna, 1960. STI-PUB/27, pp. 40-44. Abstract.

McCollum, J. P., and J. Skok. Radiocarbon Studies on the Translocation of Organic Constituents into Ripening Tomato Fruits. Proc. Am. Soc. Hort. Sci. 75 611-616 (1960).

McIlrath, W. J., J. A. deBruyn, and J. Skok. Influence of Boron Supply on the Micronutrient Element Content of Setaria Shoots. Soil Science 89 117-121 (1960).

Nebel, B. R. On the Structure of Mammalian Chromosomes During Spermatogenesis and after Radiation with Special Reference to Cores. Fourth International Conference on Electron Microscopy, Berlin, September 10-17, 1958, eds. W. Bargmann, D. Peters and C. Wolpers. Berlin: Springer-Verlag, 1960. pp.227-230.

Nebel, B. R., and C. J. Murphy. Comparative Effects of Elevated Temperature and 1000 r X-Radiation on the Seminal Epithelium of the Mouse. Cytologia 25 244-255 (1960).

Nebel, B. R., and C. J. Murphy. Damage and Recovery of Mouse Testis after 1000 r Acute Localized X-Irradiation, with Reference to Restitution Cells, Sertoli Cell Increase, and Type A Spermatogonial Recovery. Radiation Research 12 626-641 (1960).

Nebel, B. R., C. J. Murphy, and H. J. Linder. Autoradiographic Study with Tritiated Thymidine of Mouse Testis after 320 r and after 1000 r of Acute Localized X-Irradiation. Radiation Research 13 126-136 (1960).

PUBLICATIONS (Cont'd.)

Nebel, B. R., and D. J. McWhinnie. Effect of Chronic Gamma Irradiation on the Mouse Testis. Radiation Research 12 458 (1960). Abstract.

Nebel, B. R., S. A. Tyler, and C. J. Murphy. A Note on the Statistical Determination of Shape of Chromatin Elements in Human Sperm. J. Biophys. Biochem. Cytol. 7 377-380 (1960).

Norris, W. P., and P. E. Jenkins. Epoxy Resin Embedding in Contrast Radioautography of Bones and Teeth. Stain Technol. 35 253-260 (1960).

Patt, H. M. Chemical Approaches to Radiation Protection in Mammals. Fed. Proc. 19 549-553 (1960).

Patt, H. M., E. M. Jackson, and J. H. Pomeroy. "Pentoxyl" and Radiation Neutropenia. Radiation Research 12 461-462 (1960). Abstract.

Patt, H. M., M. A. Maloney, and C. L. Weber. Radiation Effects on Neutrophil Balance. Radiation Research 12 462 (1960). Abstract.

Paul, A. G., N. J. Scully, and A. E. Schwarting. The Biosynthesis of C^{14}-Labeled Ergot Alkaloids. J. American Pharmaceutical Association 49 14-16 (1960).

Pizzarello, D. J., W. E. Kisieleski, and H. Lisco. Metabolic and Autoradiographic Studies of 3-Methylcholanthrene Labeled with Tritium and Carbon-14 after Oral Administration in Rats. Proc. Am. Assoc. Cancer Research 3 142 (1960). Abstract.

Pomeroy, J. H. Drying IR Cells: Handling Spectra. The Perkin-Elmer Instr. News 11 (3) 12 (1960).

Pomeroy, J. H., and C. A. Craig. Synthesis of Nitriles by Reaction of Aldehydes with O-N-bis(trifluoroacetyl)-hydroxylamine. Am. Chem. Soc., Div. Org. Chem. 14 90 (1960) Abstract.

Powers, E. L., and B. F. Kaleta. An Isotope Effect in the Radiation Biology of Bacillus megaterium. Biophys. Soc. Abstracts # N5: 36 (1960).

PUBLICATIONS (Cont'd.)

Powers, E. L., and B. F. Kaleta. Reduction of Radiation Sensitivity of Dry Bacterial Spores with Hydrogen Sulfide. Science 132 959-960 (1960).

Powers, E. L., and B. F. Kaleta. The Mechanism of Action of Compounds That Protect Against Radiation Damage in Bacterial Spores. Radiation Research 12 464 (1960). Abstract.

Powers, E. L., R. B. Webb, and C. F. Ehret. Storage, Transfer and Utilization of Energy from X-Rays in Dry Bacterial Spores. Radiation Research Suppl. 2 94-102 (1960).

Powers, E. L., R. B. Webb, and B. F. Kaleta. Oxygen and Nitric Oxide as Modifiers of Radiation Injury in Spores of Bacillus megaterium. Proc. Natl. Acad. Sci. 46 984-993 (1960).

Rosenthal, M. W. Radioisotope Absorption and Methods of Elimination Factors Influencing Elimination from the Body. Symp. on Radioisotopes in the Biosphere. ed. R. S. Caldecott and L. A. Snyder. Minneapolis: Univ. Minn., 1960. pp. 541-563.

Roth, L. E. An Electron Microscope Study of Food Vacuoles in Pelomyxa. J. Protozool. 7 176-185 (1960).

Roth, L. E. Observations on Division Stages in the Protozoan Hypotrich Stylonychia. Proc. 4th Intern. Congr. Electron Microscopy, Berlin, September 10-17, 1958, eds. W. Bargmann, D. Peters, and C. Wolpers. Berlin: Springer-Verlag, 1960. pp. 241-244.

Roth, L. E. Observations on Cells of the Ovotestis of a Pulmonate Snail. Proc. 4th Intern. Congr. Electron Microscopy, Berlin, September 10-17, 1958, eds. W. Bargmann, D. Peters, and C. Wolpers. Berlin. Springer-Verlag, 1960. pp. 238-240.

Roth, L. E., and E. W. Daniels. Infective Organisms in the Cytoplasm of Amoeba Proteus. J. Protozool. 7 (Suppl.) 10 (1960). Abstract.

Roth, L. E., S. W. Obetz, and E. W. Daniels. Electron Microscopic Studies of Mitosis in Amoebae. I. Amoeba Proteus. J. Biophys. Biochem. Cytol. 8 207-220 (1960).

PUBLICATIONS (Cont'd.)

Sacher, G. A. Analysis of Life Tables with Secular Terms. The Biology of Aging. Am. Inst. Biol. Sci. Symp. No. 6, 1960. pp. 253-257.

Sacher, G. A. Problems in the Extrapolation of Long-Term Effects from Animals to Man. Conf. on Estimation of Long-Term Effect of Emergency Radiation Exposure in Man, Operations Research Office, Walter Reed Hospital, Oct. 28-29, 1959. Baltimore: John Hopkins Press, 1960. pp. 3-10.

Sacher, G. A. Relation of Lifespan to Brain Weight and Body Weight in Mammals. Ciba Foundation Colloquia on Ageing, Vol. 5, The Lifespan of Animals, Eds. G. E. W. Wolstenholme and C. O'Connor. Boston: Little, Brown and Co., 1959, pp. 115-133.

Sacher, G. A. Relation of Lymphoma Incidence to Lymphocyte Count in Mice under Prolonged Daily Gamma Irradiation. Radiation Research 12 468 (1960). Abstract.

Sacher, G. A. The Dimensionality of the Life Span. The Biology of Aging Am. Inst. Biol. Sci. Symp. No. 6, 1960. pp. 251-252.

Sacher, G. A. The Effect of Physiologic Fluctuations on Mortality and Aging. The Biology of Aging. Am. Inst. Biol. Sci. Symp. No. 6, 1960. pp. 246-250.

Sacher, G. A., and E. Trucco. Some Preliminary Considerations on the Theory of Turning-Over Cellular Populations. Biophys. Soc. Abstracts #J6: 24. 1960.

Schlenk, F., and S. K. Shapiro. Tracer Studies of Methionine Metabolism. Oklahoma Conference - Radioisotopes in Agriculture, April 2-3, 1959, Stillwater, Oklahoma. USAEC Report TID-7578, pp. 61-68.

Schubert, J. Beryllium. Encyclopedia of Science and Technology. New York: McGraw-Hill Book Co., Inc., 1960.

Schubert, J. Protection and Treatment. Fallout, ed. J. M. Fowler. New York: Basic Books, Inc., 1960. pp. 115-123.

PUBLICATIONS (Cont'd.)

Schubert, J. Radiation Protection Criteria and Standards. Selected Materials on Radiation Protection Criteria and Standards: Their Basis and Use. Joint Committee on Atomic Energy, 86th Congress of the United States. Washington, D. C.: U. S. Government Printing Office. 1960. pp. 392-403.

Schubert, J. The Relationship of Metal-Binding to Salicylate Action. Proc. Conf. on Metal Binding in Medicine, Philadelphia, May, 1959. pp. 325-328 (1960).

Schubert, J., G. Anderegg, and G. Schwarzenbach. Komplexone XXXI. N-(O-Hydroxycyclohexyl)-Athylendiamin-N, N'-Triessigsaure (OETA). Helvetica Chimica Acta 63 (1) 410-413 (1960).

Schubert, J., and J. F. Fried. Chelating Agents in the Treatment of Poisoning by Polymerizable Radioelements. Nature 185 551 (1960).

Schubert, J., and A. Lindenbaum. The Mechanism of Action of Chelating Agents on Metallic Elements in the Intact Animal. Proc. Conf. on Metal Binding in Medicine, Philadelphia, May, 1959. pp. 68-74 (1960).

Shapiro, S. K. The Utilization of Sulfonium Compounds by Micr oorganisms. Bacteriol. Proc. 1960, p. 190. Abstract

Shapiro, S. K., and F. Schlenk. The Biochemistry of Sulfonium Compounds. Advances in Enzymology, ed. F. F. Nord. New York. Interscience Publishers. 1960. Vol. 22. pp. 237-280.

Shigemura, Y, and S. A. Gordon. The Conversion of Indoleacetaldehyde to Indoleacetic Acid by Enzyme Systems of Avena Seedling Coleoptiles. Plant Physiol. 35 (Suppl.) 28 (1960). Abstract.

Skok, J., H. Dyer, and N. J. Scully. Photoperiodic Behavior of Sunflower. Botan. Gaz. 121 50-55 (1959).

Skok, J., and J. McCollum. Radiocarbon Studies on the Movement of Organic Materials into Tomato Fruits. Proc. Am. Soc. Horticultural Sci. 75 611-616 (1960).

Slatis, H. M. An Analysis of Inbreeding in the European Bison. Genetics 45 275-287 (1960).

PUBLICATIONS (Cont'd.)

Slatis, H. M. Radiation-Induced Recessive Mutations on Mice. Genetics Research, Division of Biology and Medicine. TID-4041 (1960), p. 8 Abstract.

Slatis, H. M. Studies in Human Genetics. Genetics Research, Division of Biology and Medicine. TID-4041 (1960), p. 9. Abstract.

Smith, D. E. Failure of Cysteine Given Postirradiation to Protect the Hibernating Ground Squirrel. Radiation Research 12 79-80 (1960).

Smith, D. E. Space Hazards. Fed. Proc. 19 40-42 (1960).

Smith, D. E., Y. S. Lewis, and G. Svihla. Cinephotomicrography of Living Mast Cells under the Influence of Pharmacological Agents. Anat. Record 136 359 (1960). Abstract.

Speckman, T. W., and W. P. Norris. The Effect of Age at Administration upon the Retention of Single Administration of Strontium. Radiation Research 12 474-475 (1960). Abstract.

Stearner, S. P., M. H. Sanderson, E. J. Christian, and S. A Tyler. Factors Influencing the Initial Lethal Responses of the Irradiated Chick. Radiation Research 12 475 (1960). Abstract.

Stearner, S. P., M. H. Sanderson, E. J. Christian, and S. A. Tyler. Modes of Radiation Death in the Chick Embryo. I. Acute Syndromes. Radiation Research 12 286-300 (1960).

Stroud, A. N., A. M. Brues, and B. R. Svoboda. Change in Chromosome Number of Krebs-2 Mouse Ascites Carcinoma During Heterologous Growth. Proc. Am. Assoc. Cancer Research 3 153 (1960). Abstract.

Stroud, A. N., A. M. Brues, and B. R. Svoboda. DNA Synthesis of Monkey Kidney Cells in Tissue Culture after Periodic Doses of Irradiation. Radiation Research 12 476 (1960). Abstract.

Stryer, L. Energy Transfer in Proteins and Polypeptides. Radiation Research Suppl. 2 432-451 (1960). Abstract.

PUBLICATIONS (Cont'd.)

Stryer, L., and E. R. Blout. Determination of the Relative Sense of the Helix in Polypeptides. Biophys. Soc. Abstracts #H3: 17. 1960.

Sussdorf, D. H. Effect of Appendix-Shielding During Body X-Irradiation on Splenic White-Pulp Recovery in Hemolysin-Forming Rabbits. Fed. Proc. 19 198 (1960). Abstract.

Sussdorf, D. H. Repopulation of the Spleen of X-Irradiated Rabbits by Tritium-Labeled Lymphoid Cells of the Shielded Appendix. J. Infect. Dis. 107 108-114 (1960).

Sussdorf, D. H. Splenic Changes During Hemolysin Formation in X-Irradiated and Nonirradiated Rabbits. J. Infect. Dis. 105 238-252 (1959).

Svihla, G., and F. Schlenk. S-Adenosylmethionine in the Vacuole of Candida utilis. J. Bacteriol. 79 841-848 (1960).

Svihla, G., and F. Schlenk. Studies on the Vacuole of Candida utilis. Bacteriol Proc. 1960, p. 54. Abstract.

Svihla, G., F. Schlenk, and J. L. Dainko. Some Effects of Ultraviolet Irradiation on Yeast Cells (Candida utilis). Radiation Research 13 879-891 (1960).

Swick, R. W., and H. G. Wood. The Role of Transcarboxylation in Propionic Acid Fermentation. Proc. Natl. Acad. Sci. U.S. 46 28-41 (1960).

Tahmisian, T. N., and S. S. Brar. Cumulative Irradiation of Sequoia gigantea. Radiation Research 12 478-479 (1960). Abstract.

Taliaferro, W. H., and B. N. Jaroslow. The Restoration of Hemolysin Formation in X-Rayed Rabbits by Nucleic Acid Derivatives and Antagonists of Nucleic Acid Synthesis. J. Infect. Dis. 107 341-350 (1960).

Taliaferro, W. H., and L. G. Taliaferro. The Intercellular Transfer of Hemolysin. Science 132 1500 (1960). Abstract.

Thomson, J. F. Fumarase Activity in D_2O. Arch. Biochem. Biophys. 90 1-6 (1960).

PUBLICATIONS (Cont'd.)

Thomson, J. F. Physiological Effects of D_2O in Mammals. Ann. N.Y. Acad. Sci. 84 736-744 (1960).

Thomson, J. F., and F. J. Klipfel. Some Effects of D_2O in Vivo and in Vitro on Certain Enzymes of Rat Tissues. Biochem. Pharmacol. 3 283-288 (1960).

Thomson, J. F., and F. J. Klipfel. Studies on the Enzymic Dehydrogenation of Deuterated Succinate. Biochim. Biophys. Acta 44 72-76 (1960).

Thomson, J. F., and T. R. Sato. Effect of Various Inorganic Phosphorus Compounds on Oxidative Phosphorylation. Arch. Biochem. Biophys. 89 139-142 (1960).

Trucco, E. Note on a Linear System of Differential Equations. Bull. Math. Biophys. 22 169-180 (1960).

Turner, J. Tracer Studies of S-Methylmethionine Homocysteine Transmethylase in Higher Plants. Fed. Proc. 19 7 (1960). Abstract.

Tyler, S. A., R. Baserga, P. B. Putong, and W. B. Wartman. The Dose-Response Relationship between the Number of Embolic Tumor Cells and the Incidence of Blood-Borne Metastases. Brit. J. Cancer 14 173-185 (1960).

Tyler, S. A., and S. P. Stearner. A Statistical Procedure for Partitioning the Acute Radiation Response into Component Processes. Radiation Research 12 481 (1960). Abstract.

Tyler, S. A., and S. P. Stearner. Modes of Radiation Death in the Chick Embryo. II. A Model of Lethal Mechanisms. Radiation Research 12 301-316 (1960).

Vogel, H. H., Jr., and N. A. Frigerio. The Effects of Single and Repeated Whole-Body Irradiation with Fission Neutrons on the Volitional Activity of the Mouse. Anat. Rec. 137 399 (1960). Abstract.

Vogel, H. H., Jr., N. A. Frigerio, and D. L. Jordan. Prophylactic and Therapeutic Treatment of Neutron-Irradiated Mice. Radiation Research 12 483-484 (1960). Abstract.

PUBLICATIONS (Cont'd.)

Vogel, H. H., Jr., and D. L. Jordan. The Combined Action of Bone Marrow and Streptomycin in Protecting Neutron-Irradiated Mice from Acute Mortality. Transplantation Bull. 7 413-415 (1960).

Wassermann, F., and T. F. McDonald. Electron Microscopic Investigation of the Surface Membrane Structures of the Fat Cell and of Their Changes during Depletion of the Cell. Z. Zellforsch. 52 778-800 (1960).

Watanabe, R., and R. E. Stutz. The Effect of Gibberellic Acid and Photoperiod on Indoleacetic Acid Oxidase in Lupinus albus L. Plant Physiol. 35 359-361 (1960).

Webb, R. B., E. L. Powers, and C. F. Ehret. Thermorestoration of Radiation Damage in Dry Bacterial Spores. Radiation Research 12 682-693 (1960).