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TABLE OF CONTENTS

	<u>Page</u>
Progress report: X- and gamma-ray toxicity Douglas Grahn, Katherine F. Hamilton and George A. Sacher	5
Survival of guinea pigs given periodic gamma-ray exposure for the duration of life George A. Sacher, Douglas Grahn and Robert J. Robertson .	13
The life span of the chicken S. Phyllis Stearner and Emily J. Christian	15
Use of ammonium thiocyanate to increase the sensitivity of the ferrous sulfate dosimeter Norman A. Frigerio	17
Comparative protective value of AET-Thiogel A in gamma- irradiated and neutron-irradiated mice Donn L. Jordan and Howard H. Vogel, Jr.	19
Progress report: Longevity of mice whose mothers received single whole-body irradiation with fission neutrons Donn L. Jordan and Howard H. Vogel, Jr.	21
Progress report: Mammary tumor incidence and longevity in female Sprague-Dawley rats irradiated with fission neutrons or with Co ⁶⁰ gamma-rays (600 days after first irradiation) Howard H. Vogel, Jr. and Donn L. Jordan	22
Fertility of CF No. 1 male mice irradiated with fission neutrons or Co ⁶⁰ gamma rays Howard H. Vogel, Jr. and Donn L. Jordan	26
Progress report: Diseases and care of laboratory animals. Further studies on the diagnosis of <u>Pseudomonas aeruginosa</u> infection of mice Robert J. Flynn and Isabel Greco	28
Induction of osteosarcomas in mice by X-irradiation Miriam P. Finkel, Patricia Bergstrand-Jenkins and Birute O. Biskis	33

TABLE OF CONTENTS

	<u>Page</u>
Progress report: Toxicity and metabolism of radionuclides in dogs.	
II. Semiannual report for period ending December 31, 1961. William P. Norris, Louise S. Lombard, Carl E. Rehfeld, Thomas W. Speckman	36
Effect of nitrogen mustard on the radiorestorative component of amoeba protoplasm	
Edward W. Daniels	53
Recovery of reproductive function in supralethally X-irradiated amoebae following cytoplasmic microtransfer	
Edward W. Daniels	54
Progress report: Experimental studies on the Cu(I, II)-peroxy theory of radiobiological effects and their modification	
Biological studies	
Joan F. Fried and Jack Schubert	55
Biochemical studies	
Jack Schubert and William M. Westfall.	58
The simultaneous determination of C ¹⁴ and H ³ in biological material, using the Schöniger combustion technique	
Martinus J. Frissel and Walter E. Kisielewski	62
Silica gel structure and the chromatographic process: Surface energy and activation procedures	
Peter D. Klein	66
Some effects of ionizing radiation on methionine sulfonium compounds	
Fritz Schlenk and Julia L. Dainko	75
Progress report: The biosynthesis of methionine	
Utilization of S-adenosylmethionine by microorganisms	
Stanley K. Shapiro	84
Progress report: Propionic acid metabolism	
III. The conversion of acrylate to succinate	
Robert W. Swick and Isabelle S. Waldron	90

TABLE OF CONTENTS

	<u>Page</u>
Effects of deuterium on malic dehydrogenase John F. Thomson, David A. Bray and Jacqueline J. Bummert	96
Effects of bubbling on cell suspensions Robert B. Webb	100
Glycerol and water effects on X-ray sensitivity in <u>Staphylococcus aureus</u> : Evidence for surface adsorption Robert B. Webb	104
Electron microscopy of mitosis in the giant amoeba, <u>Pelomyxa illinoisensis</u> Edward W. Daniels	112
Electron microscopic observations of the effect of X-irradiation on the cerebral cortex of the rat Thomas F. McDonald and F. Wassermann	115
Low temperature storage of epoxy resins used for embedding in electron microscopy O. T. Minick	121
The fine structure of meiotic chromosomes in the pigeon Bernard R. Nebel and Elizabeth M. Coulon	123
The structure of spindle fibrils in a giant amoeba L. Evans Roth and Edward W. Daniels	126
A method for obtaining fixing solutions osmotically equivalent to physiological saline solution Theodore N. Tahmisian	127
Electron microscopic examination of the wall of the lacunae and canaliculi in bone F. Wassermann	129
Radioautographic study concerning the role of ascorbic acid in collagen fiber formation F. Wassermann, Walter E. Kisielecki and Lucille Agemura . .	139
Action spectra and quantum requirements for photosynthetic processes Martin Gibbs, Clanton C. Black, John F. Turner, David W. Krogmann, and Solon A. Gordon	142

TABLE OF CONTENTS

	<u>Page</u>
Inactivity of hexitols and hexoses in the biosynthesis of auxin from tryptophan Solon A. Gordon and Evelyn Buess.	144
Further aspects of the photoperiodic responses of <u>Sequoia gigantea</u> John Skok	146
Spectral response of phosphate metabolism in germinating lettuce seeds Kenneth Surrey	147
Gamma-ray spectroscopy in the upper atmosphere Philip F. Gustafson.	155
P ³² uptake as an indicator of tritiated thymidine toxicity Larry D. Samuels and Walter E. Kisielecki.	158
Publications	162

PROGRESS REPORT: X- AND GAMMA-RAY TOXICITY

Douglas Grahn, Katherine F. Hamilton and George A. Sacher

This report will attempt to summarize the status of several experiments that were in progress during the period 1958-1961 while the senior author was on temporary duty, AEC, Washington, D. C.

I. Late Effects of Low-Dose Duration-of-Life Gamma Irradiation

In August, 1958, a major change was instituted in the programming of the low-level gamma room. The exposure day was shortened to about $7\frac{1}{2}$ hr and a 1-in. lead shield was positioned to reduce the radiation intensity over a 135° sector of the room. The new dose rates in this sector are approximately 1.3 and 2.6 r/day, while the dose behind the $2\frac{1}{2}$ in. bulk lead shield in the southeast corner of the room is reduced to about 0.3 r/day. Previously employed dose levels of 6, 12, 24, 32, 43, 56, 74, 97 and 125 r/day are maintained with an increased animal-holding capacity at 6 r/day and a somewhat reduced capacity at the higher levels.

Three inbred strains (BALB/c, C57BL/6 and A/Jax) and one hybrid (C57BL/6 x BALB/c or BC F₁) are being exposed to doses up to 56 r/day, thus broadly overlapping the original γ -ray toxicity program. Age at entry is 100 days. At 0.3 r/day, space limitation allowed only two strains, the BALB/c and BC F₁, to be entered. The numbers of animals to be exposed are given in Table 1.

TABLE 1

Proposed sample sizes by strain and dose.
Each sample composed of equal numbers of each sex

Dose, r/day	BC F ₁	BALB/c	C57BL/6	A/Jax	Total
0	336	168	168	168	840
0.3	240	120	-	-	360
1.3	336	168	168	168	840
2.6	192	96	96	96	480
6	192	96	96	96	480
12	96	96	96	96	384
24	96	96	96	96	384
32	48	48	48	48	192
43	48	48	48	48	192
56	48	48	48	48	192

With the exception of 0.3 r/day, dose effects from 0 to 6 r/day can be directly compared across all strains and sexes or within any strain or sex subdivision since proportionality of the strain sample sizes is maintained. From 12 r/day and up, equal sample sizes are used and a double weighting of the BC F₁ in this higher dose range would permit full orthogonal comparison from 0 to 56 r/day. As it is constituted, the experiment is the single biggest low-dose continuous exposure test series ever initiated and will only be exceeded by the proposed "megamouse" studies to be carried out at ANL and ORNL. The results of the present experiment will, therefore, be most pertinent to the design considerations of the larger efforts.

Entries into the experiment are complete through 6 r/day and will soon be complete through 24 r/day. Space limitation is causing a stretch-out in completion at higher dose levels.

Table 2 presents the mean survival data by sex, dose and strain for replications that have completely died out; a replication in this experiment consists of one dozen mice/sex/strain. Figure 1 indicates the dose-response relations on the semilog plot (log MAS vs dose in r/day) used by Sacher and Grahn⁽¹⁾ to describe γ -ray exposure data from the previous duration-of-life experiment.

TABLE 2

Preliminary survival figures by sex, strain and dose
(Number of animals given in parentheses)

Dose, r/day	Sex	Mean after survival, days			
		BC F ₁	BALB/c	A/Jax	C57BL/6
0	F	799 (12)	624 (36)	503 (36)	615 (24)
	M	710 (34)	570 (24)	417 (47)	624 (24)
0.3	F	672 (12)	622 (23)	-	-
	M	694 (12)	379 (22)	-	-
1.3	F	684 (36)	593 (48)	482 (35)	623 (24)
	M	675 (36)	500 (24)	449 (47)	545 (46)
2.6	F	651 (24)	528 (24)	500 (24)	550 (24)
	M	700 (36)	443 (23)	423 (24)	562 (36)
6	F	601 (24)	466 (12)	531 (24)	583 (12)
	M	617 (24)	-	420 (24)	487 (24)
12	F	427 (12)	-	397 (11)	-
	M	526 (12)	-	387 (24)	488 (12)
24	F	314 (12)	-	224 (24)	261 (12)
	M	253 (12)	-	217 (23)	254 (24)

Figure 1. Log mean after survival as a function of daily dose of Co^{60} γ -rays.

Earlier experience had shown the BALB/c mouse to have the shortest control survival, about 400 days from the start of exposure at 100 days of age. In this series, the BALB/c strain has shown considerable improvement though it may still show the shortest survival under daily irradiation. As will be noted in Section III below, there have been changes in animal husbandry procedures to reduce infection pressure, thus improving the quality of the animals. The three longest-lived strains, BC F₁, C57BL/6 and BALB/c, all demonstrate a measurable degree of life-shortening down to the very lowest dose levels. Although this was not anticipated, the finding is nevertheless consistent with our present understanding of the phenomenon of "oversurvival" at low doses. This is usually seen in those populations that have a high control incidence of infectious disease mortality. We infer that this can somehow be moderated by low levels of radiation. The apparently better quality of the mice in this study has eliminated the tendency toward marked oversurvival.

The lack of response of the A/Jax strain mice at doses below 12 r/day may be attributed to a mechanism entirely different from one of infectious disease pressure. This strain is characterized by a high incidence of mortality from amyloidosis that appears to foreshorten life. Since low-level radiation exposure does not appreciably alter the course of events for this degenerative process [Leshner et al.⁽²⁾], this mouse may be forced into a uniformity of response that is quite independent of slowly accumulating radiation injury.

A comparison of these data with those reported in the next section emphasizes the difficulty of establishing uniform control experience and the impact this difficulty has on the interpretation of experimental data.

II. Late Effects of Whole-Body Sublethal X-irradiation

Analysis of the chronic survival data from five inbred mouse strains and one hybrid exposed to single (acute lethal) doses of 200 kvp X-rays suggested that a single chronic injury parameter could define the basic life-shortening effect of radiation in all mice.⁽³⁾ Additional exploration of this concept was deemed desirable as a concurrent experiment to the low-daily-dose series (see above).

Cages of 12 per sex and strain are subdivided into 3 groups of 4 for exposure to levels of 0 r, 100 r and 0.6 of the LD_{50/30}. A common cage environment is thus provided in each replication for the 3 dose groups. At death, the mice are grossly screened for the presence of leukemia, ovarian tumors and other major neoplasms. Equal numbers of the two sexes have been used for strains BC F₁, BALB/c, C57BL/6, A/He and C3H_f/He. The simplicity of the replicate has permitted this test to be open ended. To date, there have been 9 replications per sex for the BC F₁; 5 for the BALB/c; 9 for the C57BL/6; 11 for the A/He, and 13 for the C3H_f/He.

Survival data on completed dose groups are given in Table 3. The sex difference in life shortening, previously noted by Sacher,⁽⁴⁾ and Grahn,^(3,5) is predominant in this test series. Among the males, life-shortening at 100 r is generally small or nonexistent and consistently below that at the 0.6 LD_{50/30} dose. No effort will be made to integrate the present preliminary data with past analyses since early returns on life-time experiments are invariably biased toward short survival.

It should be mentioned that this experiment has been a heterogeneous one in certain respects. The animals are housed in room E-144, which has been something of a hard-luck room in the γ -ray toxicity program because of intermittent infectious disease outbreaks. The provision of a common cage environment has provided the advantage, however, of producing a uniform exposure of all experimental groups to this environmental stress. The unpredictable and undefined respiratory infection that characterizes this particular animal room will, for example, wash out all dose effects in afflicted cages if the disease onset is within a year of exposure. In the long run, however, this unpredictable endemic may bring forth some extremely valuable data on environmental interactions with low dose responses to radiation that again will have a bearing on the future planning of chronic toxicity experiments.

TABLE 3

Preliminary survival figures by sex, strain and dose
(Number of animals given in parentheses)

Strain	Sex	Mean after survival, days		
		0 r	100 r	0.6 LD _{50/30} *
BALB/c	F	489 (13)	437 (16)	438 (16)
	M	308 (14)	330 (14)	247 (14)
A/He	F	509 (24)	493 (32)	395 (31)
	M	452 (24)	488 (27)	438 (28)
C3H/f	F	406 (31)	420 (32)	386 (32)
	M	421 (35)	412 (36)	402 (32)
C57BL/6	F	590 (15)	520 (20)	469 (28)
	M	495 (24)	522 (20)	452 (24)
BC F ₁	F	858 (16)	664 (16)	573 (16)
	M	665 (20)	634 (16)	513 (24)

*BALB/c: 300 r; A/He: 335 r; C3H/f: 355 r;
C57BL/6: 380 r; BC F₁: 395 r.

III. Analysis of the Formal Genetics of Radioresistance

In 1957, a recurrent backcross test system was initiated for the analysis of the inheritance of differences in radioresistance between strains BALB/c (P₁) and C57BL/6 (P₂). The analysis of the P₁, P₂, F₁, F₂ and F₃ generations indicated overdominance (F₁ higher than the high parent) for the LD_{50/30} values,⁽⁶⁾ while the variances adhered to more classical concepts of simple genetic additivity. In addition, a significant association or linkage between the albino gene and sensitivity was detected in the F₂ and F₃ generations.

The BC F₁ was backcrossed to both parents to yield the BC₁ (BC F₁ × P₁) and BC₂ (BC F₁ × P₂). Recurrent backcrossing was then established with P₁ to produce generations BC_{II}, BC_{III}, BC_{IV}, BC_V and BC_{VI}. No further backcrossing to P₂ was done beyond the first generation.

The BC₁ segregated for the albino, brown agouti and black agouti coat colors. Since the BALB/c parent carries the genes for brown agouti whose expression is inhibited by albino, the BC_{II} and subsequent generations were set up with separate albino and brown agouti family lines.

Theoretically, one could thus obtain the "non-albino brown agouti 'Bagg Albino.'" This separation of families also enhanced the linkage analysis since albino continued to segregate 1:1 in the brown agouti family due to the forced heterozygous condition at the albino locus in the non-albino line.

In each generation, the $LD_{50/30}$ and dosage-mortality slope were determined. The data are still being analyzed, but first estimates of the $LD_{50/30}$ values are given in Table 4. On the assumption of additivity for the genes controlling radioresistance, the $LD_{50/30}$ for any generation should move one-half the distance between its immediate parent backcross or hybrid generation and the P_1 . Expected $LD_{50/30}$ values are given in the table as calculated on the basis of this exponential rate of change. Adherence to expectation is unusually good. Obviously, then, the original postulate of overdominance is not valid, and the data can best be explained on the basis of a transgressive inheritance hypothesis. In this, it is assumed that there are some genes for resistance in the P_1 that differ from those contributed by P_2 so that the F_1 expresses the sum of all resistance genes and moves to a position above the high parent. Implicit in this hypothesis is the assumption that resistance is dominant to sensitivity. Other examples of inter-allelic additivity combined with intra-allelic dominance are given by Falconer.(7)

TABLE 4

Provisional estimates of the $LD_{50/30}$ values by generation

Generation	$LD_{50/30}$, r	% P_1	Expected $LD_{50/30}$, r
P_1 (BALB/c)	550	100	-
F_1 (BALB/c x C57BL/6)	710	50	-
BC_I	634	75	630
BC_{II}	599	87.5	590
BC_{III}	570	93.75	570
BC_{IV}	559	96.875	560
BC_V	551	98.438	555
BC_{VI}	562	99.219	552.5

Here, too, one experimental difficulty can be mentioned. The original $LD_{50/30}$ values, determined in 1953-1955, were roughly 500 r, 630 r and 660 r for the P_1 , P_2 and F_1 . The backcross series was started in 1957 and continued through 1961. Early results indicated unexpectedly high $LD_{50/30}$ values that at first suggested the presence of a genetically nonspecific "heterozygote advantage," particularly for the brown agouti family. There was the suspicion, however, that husbandry changes between 1955 and 1958

might have changed the experimental base line. Consequently, the P_1 and F_1 mice were periodically retested during the course of the BC_{III} to BC_{VI} tests. Animal supply commitments to other programs prevented the testing of the P_2 during that time although retest has now been started. The re-examination revealed that the P_1 and F_1 $LD_{50/30}$ values both shifted upward by about 9%. The newer values are the ones given in Table 4. Though a shifting base-line can raise some doubts as to the validity of the genetic interpretations, the shift apparently occurred prior to the backcross testing and the retest values for the P_1 and F_1 mice were consistent for 5 estimates on the P_1 and 8 on the F_1 .

The analysis of the slopes and variances is not complete, and here, too, some time trends may require rationalization. The linkage analysis is also incomplete but the data provide good support for the evidence of a linkage between the albino locus and one or more major genes for sensitivity. Recombination may be in the neighborhood of 25-30%. This analysis is particularly frustrating since the linkage between a single morphological trait and a complex quantitative trait measured on an all-or-none basis appears to leave more unknowns in the formulations than can be solved by the data.

Ultimately, other aspects of the series will be studied to yield a variety of analyses that can be used to check the genetic interpretations. Three readily available measures are: mean survival time of decedents in the 30-day period, litter size, and frequency of white tail spotting.

One additional check on the data has been set up. This involves the re-establishing of the BC_1 and the crossing of it back to the F_1 to give a point that is genetically half-way between the F_1 and BC_1 ; a mouse that is 62.5% BALB/c. At the same time, the BC_1 and F_1 will be spot-tested for consistency with present data. If the $BC_1 \times F_1$ generation falls at its expected position in the genetic array, then, the authors, at least, will be relieved of the last uncertainties induced by the shifted base-line figures.

The assumption of additivity with dominance also prescribes the nature of the distribution of sensitivities within the population. The distribution will, of course, still be derived by expansion of the binomial equation, but the equation will be $(\frac{3}{4} + \frac{1}{4})^n$ where n is the number of loci involved. When no dominance exists, the equation is $(\frac{1}{2} + \frac{1}{2})^n$ and a substantially normal distribution is defined. For dominance, there is considerable negative skewing at small values of n . When n is 10 or greater, the skewing is less apparent but might be detectable in the dosage-mortality slopes when these are estimated by a large array of closely-spaced dose points. The routine 3-point test system used in the present backcross series would probably only detect major deviations from linearity, and present analyses do not indicate their existence. This at least suggests that the number of genes is probably

larger than 10. A careful re-examination of the original F_2 mortality data, which spanned a dose range of 255 r with 8 separate dose points, might be more revealing.

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SURVIVAL OF GUINEA PIGS GIVEN PERIODIC GAMMA-RAY EXPOSURE FOR THE DURATION OF LIFE

George A. Sacher, Douglas Grahn and Robert J. Robertson

The survival of guinea pigs that were entered into a low-level duration-of-life experiment about 3 to 4 years ago is summarized in Table 5.

Table 5

Survival of guinea pigs given periodic gamma exposures for the duration of life

Weekly dose, r	Treatments per week	Shipment No.	No. animals entered	No. living as of 21 Dec. 1961	No. living after 844 days	Age as of 21 Dec. 1961, days
0	0	1	12	7	9	1327
		3	12	5	5	1166
		4	12	5	8	1082
		5	12	7	7	984
Subtotal			48	24	29	
0	1	2	12	6	9	1243
		4	12	8	8	1082
Subtotal			24	14	17	
0	4	1	12	6	9	1327
		2	12	5	6	1243
		3	12	9	11	1166
		5	12	10	10	984
Subtotal			48	30	36	
Grand total			120	68	82	
1.35	1	1	32	15	27	1327
		2	30	9	17	1243
		3	18	11	11	1166
		4	8	3	4	1082
		5	8	6	6	984
Subtotal			96	44	65	
5.4	4	1	16	8	14	1327
		2	16	9	11	1243
		3	8	6	7	1166
		5	8	8	8	984
Total			48	31	40	
13	5	1	16	3	9	1327
		2	14	6	11	1243
		4	10	3	3	1082
Total			40	12	23	
24	4	2	4	0	0	1243
		3	4	0	1	1166
		5	4	1	1	984
Subtotal			12	1	2	
24	1	2	4	0	0	1243
		3	4	0	1	1166
		5	4	1	1	984
Subtotal			12	1	2	
Grand total			24	2	4	
36	6	1	8	0	5	1327
			8	0	1	1082
Total			16	0	6	

There are 5 exposure levels, ranging from 1.35 r/week to 36 r/week of Co^{60} γ -rays. In this experiment, the exposed groups do not remain in the gamma exposure room. They are moved into the gamma room, receive their scheduled number of days of exposure and are then returned to the animal quarters for the remainder of the week. Thus every treatment group spends from 1 to 6 days in close proximity to the control pigs. In addition, the controls are divided into subgroups, each of which has a movement pattern approximating that one of the exposure groups.

The controls and the two lowest exposure levels have more than 50% survival, so a detailed discussion of the survival will be postponed to the next report. Some trends are appearing, which can be briefly noted, however.

1. The higher levels of exposure, 24 and 36 r/week, are extremely lethal to the pigs. This confirms the findings of Lorenz et al.⁽¹⁾ on guinea pigs given 4.4 and 8.8 r/day for the duration of life.

2. There is as yet no evidence that 1.35 or 5.4 r/week have produced excess mortality. In fact, these two groups, with over 150 weeks of accumulated exposure, have a survival experience which is better than control, though not significantly better.

3. The 13 r/week group is showing accelerated mortality in the third year of exposure. This also confirms the results of Lorenz et al.⁽¹⁾

Lorenz et al. did not have large enough samples to permit estimation of the life shortening at daily doses of 1.1 or 0.11 r/day. The present experiment was set up to determine whether the extreme radiosensitivity observed by Lorenz at 2.2 r/day and above continues to be present at exposure levels below 1 r/day. As space becomes available, more pigs will be entered at 5.4 r/week. In the past year, 24 were added at this level. They receive 2.6 r/day in 2 consecutive days, and have an equal number of moving controls.

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THE LIFE SPAN OF THE CHICKEN

S. Phyllis Stearner and Emily J. Christian

In studies of ionizing radiation effects on the life span of animals, investigations of normal longevity usually must be included. Reliable survival records are available for very few species of mammals and are virtually nonexistent for other classes of vertebrates.⁽¹⁻³⁾ In birds there is a small body of information on wild and zoo populations, while data from flocks of domestic poultry usually include only portions of the life span. In this laboratory, a control group of White Leghorn roosters is being maintained for duration of life, as part of a long-term study of radiation effects in the chicken.

The birds were obtained as newly-hatched chicks from Kerr Chickeries, Frenchtown, New Jersey. Roosters and hens derived from a single flock have served as the source for all eggs. Between December 1952 and June 1960, a total of 87 cockerels obtained from several shipments have been put into the experiment, as space in the animal quarters was available. At 30 days of age,* the chicks were numbered and

weighed. They were then caged individually and maintained under conditions standard for our animal quarters. At regular intervals, weights were taken and general condition noted. Gross changes were recorded at autopsy, but histopathology is not yet available. Several groups have now exceeded 6 years of age. Survival over this period (2190 days) is reported here.

Figure 2. Survival of untreated White Leghorn roosters through 6 years, based on the number alive at 30 days.

Survival per 30-day period is shown in Figure 2. There is a high juvenile mortality, but throughout the adult period up to 2000 days the survival curve approximates an arithmetic straight line. There is 50% survival at 2050 days. After 2000 days there is indication of an increase in mortality. We do not know now if this trend will continue, for relatively few birds have been observed for more than 6 years.

Based on records of the oldest roosters in the flock (9 years), however, a pronounced tail on the survival curve is to be expected with the last half-decade surviving for extremely long periods.

*Mortality within the first 30 days averaged less than 3%.

During the juvenile period mortality is largely associated with lymphomatosis. But even after the juvenile period, it continues to be the most common single finding at autopsy

We have found one report in the literature of survival in White Leghorn hens over a 10-year period.⁽⁴⁾ The annual mortality rate for a flock of 200 was recorded in connection with a study of egg production, with (apparently) no culling. The survival curve through 1 to 10 years is linear on arithmetic coordinates. There was 50% survival at 7 years and 13% survived at 10 years when the study was terminated. These values are based on 100% survival at the start of observations, presumably about 6 months.

The straight-line survival curve has been described by Comfort for zoo populations of some mammals and for the peafowl.⁽²⁾ The absolute number dying per unit time is constant; the mortality rate increases with age, and there is no commonest age of adult death. This is in contrast to the log-linear decline in survival usually found in wild populations. In zoo animals, a higher mortality may be expected from epidemics and increased territorial fighting, but these animals have advantages such as adequate food supply and absence of predators. Our laboratory population of roosters is protected from fighting and predators. Virus infections associated with the leucosis complex represent the most prevalent disease. Lack of exercise and nonspecific stress associated with confinement in small cages may act to decrease life expectancy in laboratory populations such as this one, and perhaps even in zoo animals, but the nature of such influences is not understood. In the absence of complete life tables on any species of birds in captivity, there is no information bearing on the relation of aging to development or even on whether aging occurs as it does in mammals.

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USE OF AMMONIUM THIOCYANATE TO INCREASE THE SENSITIVITY OF THE FERROUS SULFATE DOSIMETER

Norman A. Frigerio

Ferrous sulfate dosimetry has proven very useful for the measurement of doses in the region 4000 to 40,000 r, and various techniques and sensitizing agents have been successfully employed to extend the lower limit to 500 r.⁽¹⁾ However, even this dose level is above that of interest for many biological effects, particularly with neutrons. We have found that addition of a solution of NH_4SCN to an exposed ferrous sulfate dosimeter increases the spectrophotometric sensitivity by a factor of 6.

The intense red color of the ferric thiocyanate complex is quite stable as a function of time and can be measured in a simple visible colorimeter without recourse to an ultraviolet spectrophotometer. It is linearly dependent on the ferric ion concentration and is unaffected by the presence of sensitizing agents so that these may be employed to further increase the overall sensitivity of the system if desired.

The increased sensitivity results from the increased extinction coefficient of the ferric thiocyanate complex $[\text{Fe}(\text{SCN})_6]^\equiv$, $\epsilon_{475} = 17,600$ liters/mole-cm] compared to that of ferric ion alone (Fe^{+++} , $\epsilon_{304} = 2187$ liters/mole-cm).

Ferrous-ortho-phenanthroline also possesses a high extinction coefficient ($\epsilon_{508} = 11,400$ liters/mole-cm). It is not useful in the low-dose range, however, because of the difficulty of precisely measuring small decrements of ferrous ion in the presence of a high ferrous ion concentration.

The solution used was 10^{-3} M in $\text{Fe}(\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$, 10^{-3} M in NaCl and 0.4 M in H_2SO_4 made up in triply quartz-distilled water. When sensitization was required, benzoic, phthalic or salicylic acid was added to a concentration of 1.5×10^{-3} M. Irradiation was accomplished with a Co^{60} source that had been standardized with a standard FeSO_4 dosimeter and with National Bureau of Standards calibrated Victoreen chambers. The dose rate was 4 r/min.

When irradiation was complete, 3 ml of the dosimeter solution were measured into a 1-cm quartz cuvette. Readings of the irradiated solution and of an unirradiated blank were first made at 304 m μ . If the dosimeter had received 4000 r or more this was sufficient for dose measurement. For lower doses, 1.0 ml of saturated NH_4SCN solution was added and the contents of the cuvette were mixed by inversion. The sample was then read again at 475 m μ .

Although the extinction coefficient of $\text{Fe}(\text{SCN})_6^{\equiv}$ is 17,600 liters/mole-cm, the dilution attending the addition of the saturated NH_4SCN solution resulted in an effective ϵ_{475} of only 13,200 liters/mole-cm. The absorbancy at 475 $\text{m}\mu$ so obtained proved perfectly linear over the range 10-10,000 r. The same ϵ_{475} and dose linearity were obtained with FeSO_4 solutions to which sensitizers had been added.

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COMPARATIVE PROTECTIVE VALUE OF AET-THIOGEL A IN GAMMA-IRRADIATED AND NEUTRON-IRRADIATED MICE

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

A recent investigation⁽¹⁾ showed the protective effect of AET-Thiogel A in mice irradiated with supralethal doses of X-rays (800-1600 r). Absence of mortality in the early postirradiation period, usually associated with the intestinal syndrome, was demonstrated following this chemical protection.

Thiogel A contains 14.2 SH groups per 100,000 molecular weight. Thus it might add to the protective affects of AET alone. Several experiments were carried out in the acute lethal dose range with this mixture for comparison with previous work on the protective effect of AET alone.⁽²⁾

Groups of CF No. 1 female mice were irradiated with single doses of fission neutrons and Co^{60} γ -rays in the acute lethal range. Irradiated controls received no pretreatment. Experimental animals received 1 ml of solution containing 8.5 mg AET and 100 mg Thiogel A administered by oral injection. Half of the injection was given approximately 20 minutes before and the other half immediately before irradiation.

All exposures were carried out at the CP-5 reactor in the neutron-gamma radiation chamber.

Figure 3 shows the dose-mortality relationship of animals irradiated with Co^{60} γ -rays. Each point represents at least 12 mice. The $\text{LD}_{50/30}$ of the untreated irradiated controls was approximately 830 rads, whereas that of the γ -irradiated mice pretreated with AET-Thiogel was approximately 980 rads. This represents a dose reduction of 19%.

Figure 4 shows a similar plot of dose vs mortality for fission neutrons. The $\text{LD}_{50/30}$ for the untreated control group was approximately 323 rads in contrast to 353 rads for treated animals. This dose reduction is on the order of 9%, only one half as much as for γ -irradiation.

Latarjet et al.⁽¹⁾ showed that the AET-Thiogel mixture selectively protected the intestine in mice given supralethal doses of γ -rays. However, the use of Thiogel A prior to neutron irradiation did not appear to increase the protective effect of preadministration of AET alone.

Figure 3. Dose-mortality relationship of CF No. 1 female mice exposed to Co⁶⁰ gamma-rays. Upper line, irradiated controls; lower line, pretreated with 8.5 mg AET and 100 mg Thiogel A.

Figure 4. Dose-mortality relationship of CF No. 1 female mice exposed to fission neutrons. Upper line, irradiated controls; lower line, pretreated with 8.5 mg AET and 100 mg Thiogel A.

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PROGRESS REPORT: LONGEVITY OF MICE WHOSE MOTHERS
RECEIVED SINGLE WHOLE-BODY IRRADIATION WITH
FISSION NEUTRONS

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

The 500-day cumulative mortality of the progeny from CF No. 1 females exposed to single doses of fission neutrons does not appear to differ significantly from the mortality of the progeny of unirradiated controls. Table 6 shows the mortality of the neutron-irradiated and unirradiated control animals. In each group, 78% have survived the 500-day period.

TABLE 6

500-Day mortality of progeny of CF No. 1 female mice irradiated
with small doses of fission neutrons

	Progeny from control mothers	Progeny from neutron-irradiated mothers (single, whole-body dose)				
		Dose (rads)				
		20	50	75	100	100
Number weaned	245	503	138	106	56	19
Number dead at 500 days	54	102	33	27	14	3
Cumulative mortality, %	22	22	20	24	25	16

PROGRESS REPORT: MAMMARY TUMOR INCIDENCE AND LONGEVITY
IN FEMALE SPRAGUE-DAWLEY RATS IRRADIATED WITH
FISSION NEUTRONS OR Co^{60} GAMMA RAYS (600 DAYS
AFTER FIRST IRRADIATION)

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

In this report we are comparing the incidence of mammary tumors in female Sprague-Dawley rats given four whole-body 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).^(1,2) Similarly, the 55 rats exposed to Co^{60} γ -rays were divided into four dose-rate groups.

Figure 5 indicates the mammary tumor incidence in both groups of irradiated rats and in their unirradiated controls. Mammary tumors did not appear in the unirradiated rat to any appreciable extent before 15 months; however, the incidence rose sharply at about 450 days and approached 40% by 600 days. It is clear that with the radiation schedule used here approximately 90% of the rats developed mammary tumors within 20 months of the first exposure. Fission neutrons appear to be at least twice as effective as Co^{60} γ -rays in promoting the tumors.

Figure 5. Mammary tumor incidence in female Sprague-Dawley rats exposed to four weekly doses of external radiations.

Of the 112 irradiated rats, only 2 animals exposed to γ -rays and 6 exposed to neutrons died during this period; there were no observable neoplasms of the mammary glands.

In Figure 6 survivorship curves are drawn for the three groups of rats up to 600 days after the first exposure. Although the total neutron dose was only half that of the γ -rays, mortality was higher in the neutron-

irradiated rats during the experimental period. There was no appreciable mortality among the unirradiated controls during the first 500 days, and at the time of this report (600 days), less than 15% had died. Little difficulty has been experienced with the common respiratory infections that often cause greatly increased mortality in many rat colonies after the first year. In the experiments of Hartwig et al.⁽³⁾ the average lifetime for the irradiated female Sprague-Dawley rat was only 511 days, and only about half lived beyond 400 days.

Figure 6. Survivorship of irradiated and control rats during 600-day period.

It is interesting to compare the cumulative percentage of rats with one or more mammary tumors following a single whole-body exposure. Shellabarger, Bond, and Cronkite,⁽⁴⁾ using the same strain of female rats, reported a cumulative percentage of approximately 50 after a single dose of 200 r and 90% after 400 r of Co⁶⁰ γ-rays, during the 20-month period following irradiation. These authors also reported apparent mean life spans of approximately 15 months after a single exposure of 200 r, and 14 months after 400 r of γ-rays. In our experiment, the mean life span was approximately 14½ months after four weekly γ-ray exposures of 200 rads each, and approximately 11 months after the four exposures of 100 rads of fission neutrons.

In Table 7 data are presented for the mean after-survival times of the neutron- and γ-irradiated rats at the four different intensities used. These data are still preliminary since a few irradiated rats are still living. It is clear that the γ-irradiated rats live longer than the neutron-exposed animals, even though the γ-dose was twice as high. There does not at present appear to be a significant dose-rate effect on mean after-survival times although the number of animals in each sub-group may be too low for a definite conclusion. At the present time, 600 days after the first exposure, less than 15% of the unirradiated control animals have died. Further analysis will be undertaken at the conclusion of the experiment.

TABLE 7

Comparison of mean after-survival times of neutron- and gamma-irradiated rats in which the exposures were carried out at different dose-rates

Dose rate, rad/min	Number of rats	Mean after survival, days
Neutron-irradiated rats (100 rads x 4)		
1.5	14	304.1
2	14	357*
6	14	328.2
35	15	363.1
Co ⁶⁰ γ -irradiated rats (200 rads x 4)		
1	14	445*
3	13	390*
6	14	435.8
35	14	463*

*Estimated mean after-survival times at 600 days; 1 neutron-irradiated and 7 γ -irradiated rats still alive.

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FERTILITY OF CF NO. 1 MALE MICE IRRADIATED WITH FISSION NEUTRONS OR Co^{60} GAMMA RAYS

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

Several groups of CF No. 1 male mice were irradiated with small doses of Co^{60} γ -rays or fission neutrons. A week following exposure these males were placed in cages containing 3 to 4 unirradiated young adult female mice. The females were checked daily and when found pregnant were isolated. Following birth and weaning of their young, they were returned to their original cages. The time interval between the first two fertility tests was, therefore, about 6 weeks.

In these tests (Table 8) there was little difference between the irradiated and control groups either in the percentage of pregnancies observed or in the average litter size. This result contrasts with similar tests made with neutron-irradiated females at 50 rads: The second test for females showed a small change in percent pregnancy (87.5% to 75%) but a significant drop in the average litter size (7.6 to 3.7).⁽¹⁾

TABLE 8

Effects of small doses of fission neutrons and Co^{60} gamma rays on fertility of CF No. 1 male mice

Dose rads	Test #1		Test #2	
	% pregnancies	Average litter size	% pregnancies	Average litter size
0	100	11.4	92	10.9
Co^{60} γ -rays				
46	100	11.1	75	10.9
100	100	10.9	88	12.6
150	100	8.2	80	10.8
Fission neutrons				
5.6	100	10.6	73	8.1
12	92	8.5	83	9.6
19	100	9.3	91	9.9
47	100	6.0	88	9.3

The results of these first two fertility tests of males are interesting in view of the sensitivity of male germ cells to doses of X-rays.⁽²⁾ The tests will continue over the normal reproductive span of these mice.

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PROGRESS REPORT: DISEASES AND CARE OF LABORATORY ANIMALS
Further Studies on the Diagnosis of Pseudomonas Aeruginosa
Infection of Mice

Robert J. Flynn and Isabel Greco

Because Pseudomonas aeruginosa infection interferes with radio-biological research, it is desirable to eliminate this infection from the experimental animals. A rapid, accurate method of diagnosis is an essential prerequisite for any effective control program. An earlier report⁽¹⁾ described attempts to detect P. aeruginosa by means of differential growth characteristics on several bacteriological culture media. It was concluded that the practical diagnosis of this infection depended upon the detection of the one unique characteristic, pyocyanin production. The present report describes studies to determine 1) the best medium to use for pyocyanin production, and 2) the best specimen to use to detect infected animals.

Best medium. The 21 pure strains of P. aeruginosa used in these studies are listed in Table 9. Ten were isolated from mice and four from humans at Argonne National Laboratory (ANL). Three were obtained from Dr. Carolyn W. Hammond of the University of Illinois. Four were obtained from the American Type Culture Collection (ATCC).

TABLE 9

Pseudomonas aeruginosa cultures used to study pyocyanin production

Code number	Donor	Remarks
6, 43, 43A, 46, 48, 444A 444B, 444C, 443, 445	ANL* isolates	From laboratory mice
H3, AM, R1, R30	ANL* isolates	From humans
N5, AVK, HL	Hammond**	Received as non-pigmentary
257, 260, 256, 262	ATCC†	Non-pigmentary††

*Argonne National Laboratory

**University of Illinois

†American Type Culture Collection

††Haynes(2)

Most of the ANL isolates were strong pyocyanin producers. The University of Illinois isolates were supposed to be non-pigmentary but were in actuality weak pigment producers. The four from the ATCC were among those reported by Haynes⁽²⁾ not to produce pigment on the media he used (Burton's and Gessard's).

Each strain was used to inoculate both ordinary bacteriological culture media and special pigment-enhancing media (Table 10). The results obtained with the ordinary media (Figure 7) show that only the strongest pigment producers are detectable with these media.

TABLE 10

Media used for pyocyanin production

Medium	Contents
Ordinary media	
Beef heart infusion broth	Beef heart infusion
Nutrient broth	Beef extract, and peptone
Koser citrate medium + 1% BHI*	Brain infusion, beef heart infusion, proteose peptone, sodium citrate, and inorganic salts
Tetrathionate broth	Proteose peptone, bile salts, calcium carbonate, sodium thiosulfate, and iodine
Pigment-enhancing media	
Gessard's glycerol peptone agar	Glycerol (5.0%), peptone (2.0%), and agar
Sabouraud maltose agar	Maltose, neopeptone (1.0%), and agar
Burton's medium	Glycerol (1.0%), purified amino acids (1.2%), and inorganic salts
King's medium "A"	Glycerol (1.0%), peptone (2.0%), inorganic salts, and agar
Sellers' N-C-G** agar	Glycerol (1.0%), neopeptone (2.0%), sodium citrate, and agar
Wensinck's glycerol broth	Glycerol (1.0%), proteose peptone (1.5%), and inorganic salts
Wensinck's glycerol agar	Glycerol (1.0%), proteose peptone (1.5%), inorganic salts, and agar

*Brain heart infusion.

**Neopeptone-citrate-glycerol.

Figure 7

Pyocyanin production by 21 strains of *Pseudomonas aeruginosa* on ordinary media.

The results obtained when the special media were used are given in Figure 8. It can be readily seen that the pigment-enhancing media are far superior to the ordinary media for pyocyanin production. All the strains tested except the four ATCC strains produced pyocyanin on these media.

Figure 8
Pyocyanin production by 21 strains of *Pseudomonas aeruginosa* on special pigment-enhancing media.

It was, of course, possible that some of the tubes recorded as negative were actually positive but that the amount of pigment produced was so small that it was not readily discernible. For this reason the study was repeated, but this time the number of tubes inoculated from each test culture was increased from one to five. After 24 hours of incubation one of the five tubes was read for pyocyanin production, extracted with chloroform, and then read again for the chloroform-soluble pigment. The second, third, fourth, and fifth tubes were read in a similar manner on days 2, 3, 5, and 7 respectively.

In some cases chloroform extraction did indeed disclose pyocyanin production which was not discernible without such extraction (Figure 9). With Wensinck's glycerol broth, seven days' incubation, and chloroform extraction all but two of the 21 strains produced detectable amounts of pigment. It was concluded therefore that this medium and technique were best for the production and detection of pyocyanin.

Figure 9

The effectiveness of chloroform extraction in the detection of pyocyanin production. (BHI = brain heart infusion.)

Best specimen. Comparative cultures of water bottles, feces, and saliva were made. The results (Table 11) indicate that the culturing of feces and saliva from individual mice is more accurate than the culturing of single water bottle samples from these same mice. But the cumulative accuracy of multiple water bottle, fecal, and saliva tests is comparable (Figure 10). Further, the culturing of water bottle samples from cages with many mice is far more accurate than the culturing of composite fecal samples (Table 12).

TABLE 11

Results of cultures of ten mice individually isolated from
a group cage positive for Pseudomonas aeruginosa

Water bottle cultures				Saliva cultures				Fecal cultures**			
Mouse No.	Days after isolation*			Mouse No.	Days after isolation*			Mouse No.	Days after isolation*		
	1	3	7		1	3	7		1	3	7
1	+	+	-	1	+	+	+	1	+	+	+
2	-	+	-	2	+	+	+	2	-†	+	+
3	+	+	-	3	+	+	+	3	+	+	+
4	-	+	+	4	+	+	+	4	-††	+	+
5	-	-	+	5	+	+	+	5	+	+	+
6	+	+	+	6	+	-	+	6	+	+	+
7	-	-	†	7	+	+	+	7	+	+	+
8	-	-	+	8	-	+	+	8	+	+	+
9	+	+	+	9	+	+	+	9	+	+	+
10	-	-	+	10	+	+	+	10	+	+	+

*+, positive culture; -, negative culture.

**Five fecal pellets each, except where otherwise noted.

†One fecal pellet.

††Two fecal pellets.

Figure 10

Results of ante mortem and post mortem cultures of individually isolated mice from a group cage positive for Pseudomonas aeruginosa.

TABLE 12

Water bottle vs. composite fecal samples
(15 mice per cage)

Cages tested	Water bottles positive, %	Fecal samples positive, %
211	28.4	7.5

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INDUCTION OF OSTEOSARCOMAS IN MICE BY X-IRRADIATION

Miriam P. Finkel, Patricia Bergstrand-Jenkins, and Birute O. Biskis

A portion of the right hind leg of mice was exposed to X-rays, and thereafter the area was watched for the appearance of tumors. The purpose of the experiment was to determine whether X-rays were carcinogenic under the particular conditions that had been imposed. If so, the plan was to select an optimum dose to use in a series of experiments in which dose rate and pattern of fractionation would be varied. Studies of this kind with X-rays, in which the dose and many parameters of dosage can be quite precisely controlled, should be valuable aids in interpreting studies with radioisotopes, where there are many obstacles even to measuring the dose.

Materials and methods. The X-ray factors were 250 kvp, 30 ma, and filtration 3.12 mm Al. The measured dose rate at the position of the irradiated leg was 866.7 r/min. During irradiation the mice were in $\frac{1}{4}$ -inch lead chambers, which, when properly positioned, permitted the full dose to be received only by the distal quarter of the femur and proximal three-quarters of the tibia.

The mice were CF1/Anl females, about 70 days old. Four were irradiated at a time, and four groups of 44 each received 5000, 4000, 3000, and 2000 r, respectively. They were housed 14 or 15 per stainless steel cage at the beginning of the experiment, but those whose legs became sore, or who required isolation for some reason, were put in plastic cages containing wood shavings. Animals were permitted to die, or they were killed when moribund. After autopsy a skeletal roentgenogram was prepared and both hind legs were taken for histologic study.

Results to date. The data are quite incomplete at this time; one of the mice is still alive 1016 days after exposure, and most of the histopathologic work remains to be done. However, some basic information from the survival data and from the external and roentgenographic appearance of the irradiated knees can be used in planning the next stage of these experiments.

Changes in the irradiated knee ranged from slight epilation to deep ulceration and even to spontaneous amputation of a portion of the limb. The roentgenograms showed many kinds and degrees of abnormality, including osteosarcomas.

The major condition of the irradiated site of each animal at death is indicated in Figure 11. The number of cases of severe damage was greater when the dose was greater, but in individual cases the damage from

Figure 11. Frequency distribution of death and terminal lesions with time after irradiation.

2000 r was just as great as the damage from 5000 r. For example, more animals that had received 5000 r had ulcerated legs than animals that had received 2000 r, but the extent of ulceration was just as great among the latter group as among the former.

Lesions of the irradiated bones have been classified tentatively as positive, probable, or possible osteosarcomas on the basis of their roentgenographic appearance. The kind of bone damage was so different from the damage seen after the injection of bone-seeking radioisotopes that the uncertainties in diagnosis using this technique were greater than usual. The definitive diagnoses will be based on histologic examination.

Under the conditions of this experiment 5000 r seems to have been no more carcinogenic than 2000 r. Curiously,

in the 5000 r group there was only one death with tumor before 250 days after irradiation and no others until after 625 days. In the 4000 r group also the tumors appeared among the animals that died relatively late. In contrast, in the 3000 r group tumor deaths occurred with some regularity after 450 days, and in the 2000 r group after 275 days. These results give the impression that the manifestations of neoplastic change were retarded when damage was severe.

Discussion and extension of the experiment. The present data show that 2000 to 5000 r of X-ray, delivered at the rate of 866.7 r/min, are carcinogenic, but the smallest dose was not less effective than the largest. In fact, it may have been more effective. As a consequence, we do not know where within the carcinogenic range these doses fit. The impression that severe damage and tumor development are incompatible leads us to feel that the optimum carcinogenic dose lies closer to 2000 r than to 5000 r when the dose is delivered at the rate of 866.7 r/min. In fact, since severe ulceration occurred even after 2000 r, the optimum dose might be lower still.

The selection of a dose to be used in studying the effect of rate and of fractionation on the neoplastic response requires dose-response information. Another experiment that should provide this information has been started. Both hind legs of CF1/An1 female mice are being irradiated at the

rate of 58 r/min. This lower dose rate is being used in an attempt to keep non-neoplastic damage at a minimum. Three hundred mice will receive 1000 r, 750 will receive 625 r, and 750 will receive 250 r. An additional 540 untreated mice from other experiments will be available as nonspecific controls. It is hoped that this experimental plan will satisfy the statistical requirements for determining whether the results are consistent with either a linear or a square relationship between the dose and the number of osteosarcomas.^(1,2)

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PROGRESS REPORT: TOXICITY AND METABOLISM OF RADIONUCLIDES IN DOGS

II. Semiannual Report for Period Ending December 31, 1961

William P. Norris, Louise S. Lombard, Carl E. Rehfeld
and Thomas W. Speckman

This report is the second semiannual summary of our investigations of the toxicity and metabolism of selected radionuclides in pure-bred beagle dogs. To date, two fission products, Ce^{144} and Cs^{137} , have been tested to determine their acutely lethal doses ($\text{LD}_{50/30}$); and dogs were injected at lower doses of both isotopes to begin the study of their less acute effects. In addition the $\text{LD}_{50/30}$ of externally applied, whole-body Co^{60} γ -radiation was approximated experimentally. The results of these studies, and of pertinent ancillary observations, are reported in the following test.

Personnel

Eleven persons are now engaged on a full-time basis with this work; one technician was added since the last report. The breakdown of personnel is as follows: staff members, 4; technicians, 6; records clerk, 1. Dr. Herman Slatis is still participating actively in the genetics aspects of the breeding program. Drs. Henry Rosenberg and Thomas Emmering (University of Illinois, School of Dentistry) are collaborating in studies of tooth development and dental disease.

In addition, there are 3 full-time animal caretakers. This is not adequate; however, arrangements are now being made to alleviate this situation.

Physical Plant

Laboratory furniture has still not been installed and several other aspects of the primary installation are yet incomplete. This work has progressed at an exceedingly slow pace. However, prospects are now bright that this work will be done early in 1962.

Dog Colony

On the effective date of this report the total number of live beagles belonging to this program was 239. The sequential numbering system, which includes all dogs, had reached 413 (not including 8 dogs originally given numbers greater than 413). During the entire year of 1961 a total of 147 pups were born. Of these 53 were either stillborn or died within

2 weeks after birth. The usual incidence of pup mortality is actually not as high as this figure would indicate however since 30 of these 53 were derived from dams fed an experimental diet. This diet is described later in this report.

The origins of the dogs are as follows:

Cass O'Day Kennels; Murray, Utah	12
University of Utah AEC Project	14
University of California AEC Project; Davis, California	16
Dr. J. W. Coden, Alexandria, Virginia	3
Bar-B Kennels, Essex, Michigan	60*
Total born at ANL	<u>316</u>
Total	421

Inbreeding

Although the majority of the dogs are outbred, a few are being inbred in an effort to produce a uniform genotype. In the outbred population, we now encounter considerable variation between individuals. In view of the relatively small numbers of dogs which can be accommodated in an experiment, the success of the inbreeding effort should appreciably increase the statistical significance of experimental results. The progress of this work will be reviewed in detail in our next semiannual report.

Semisynthetic Dog Diet

The feasibility of feeding a semisynthetic dog food composed of casein, sugar, fat, and purified vitamins and minerals has been the subject of investigation for the past 15 months. Three test runs have thus far been made.

Three 8-week-old weanlings placed directly on the semisynthetic diet early in September 1960 are still consuming the diet and are in apparently good health. Their growth rate and performance has been comparable to that of animals receiving the stock ration. The composition of the original diet was as follows:

Sugar (sucrose)	50%
Casein	25%
Cottonseed Oil	10%
Salt Mix	10%
Cellulose	4%
Vitamin Mix	1%

The vitamin and mineral content is given in Table 13.

*These animals, referred to in the previous progress report⁽¹⁾ as the "Michigan" dogs, were mostly returned to the vendor. Some of them were used for pilot studies.

TABLE 13

Vitamin and mineral content of semisynthetic dog diet

	Quantity, g/100 g of diet
Choline chloride	0.225
Thiamine hydrochloride	0.0003
Riboflavin	0.0003
Pyridoxine hydrochloride	0.0003
Calcium pantothenate	0.0044
Nicotinic acid	0.0030
B ₁₂ - in mannitol	0.000004
Biotin	0.00002
Inositol	0.050
Folic acid	0.0002
PABA	0.0060
Menadione	0.0001
Vitamin A	600 IU*
Vitamin D	60 IU
Vitamin E	10 IU
CaCO ₃	2.78
Ca(H ₂ PO ₄) ₂ · H ₂ O	2.44
K ₂ HPO ₄	1.8
MgSO ₄	0.54
FeSO ₄ · 7H ₂ O	0.03
CuSO ₄ · 5H ₂ O	0.003
CoCl ₂ · 6H ₂ O	0.001
KI	0.0002
MnSO ₄ · H ₂ O	0.0016
ZnCl ₂	0.0011
Na ₂ SeO ₃ · 5H ₂ O	0.000003
Al(OH)(C ₂ H ₃ O ₂) _x · H ₂ O	0.00001
NaBr	0.00001
NaF	0.00001
H ₃ BO ₃	0.00001
Na ₂ MoO ₄ · 2H ₂ O	0.00001

*International Units

In November 1960 four brood bitches were placed on the semisynthetic food, bred approximately 2 months later, and carried through pregnancy and lactation on this regimen. All bitches whelped normally. However, signs of a possible vitamin E deficiency appeared in one litter shortly after weaning, at 5 weeks of age. These signs were eliminated by addition to the diet of vitamin E and a multiple vitamin preparation.

The composition of the diet was then altered: the maintenance level of protein was kept at 25%, but the level for pregnancy and lactating bitches was increased to 30%. In addition the vitamin content was increased (Table 14).

TABLE 14

Vitamin levels of semisynthetic diet for maintenance,
and for pregnancy and lactation

Vitamin	Maintenance level, g/100 g of diet	Pregnancy and lactation level, g/100 g of diet
Choline chloride	0.2250	0.2250
Thiamine hydrochloride	0.00025	0.0005
Riboflavin	0.0003	0.0005
Pyridoxine hydrochloride	0.00025	0.0005
Calcium pantothenate	0.00375	0.0060
Nicotinic acid	0.0030	0.0060
B ₁₂ - in mannitol	0.000004	0.000006
Biotin	0.00002	0.00002
Inositol	0.0500	0.0500
Folic acid	0.0002	0.0002
PABA	0.0060	0.0060
Menadione	0.0001	0.0001
Vitamin A	1000 IU*	2000 IU
Vitamin D	100 IU	200 IU
Vitamin E	25 IU	50 IU

*International Units

All animals have been maintained on these two variations of the diet since last March. All puppies born and weaned of bitches fed the synthetic diet are doing well today. Even those that showed signs of a vitamin E deficiency are thriving and show no apparent ill effects.

Late this summer four brood bitches receiving the synthetic food were again bred; two of these animals had previously delivered litters while being fed the semisynthetic food. Thus far three of these four have whelped. Unfortunately all three have had parturition difficulties and the litters were partially delivered by caesarian section. Further reappraisal of the diet is now under way in an attempt to see whether such parturition difficulties can be avoided by further dietary changes. This food certainly appears adequate for growth and maintenance. The successful development of such a diet has several attractive corollaries: 1) complete control over the composition, 2) ease of supplementation, and 3) absence of radioactivity - the only gamma radionuclide present is K⁴⁰.

Growth of Dogs

All dogs born in this colony have been weighed at regular intervals. The weight gains from birth through 13 months are summarized for males in Figure 12 and for females in Figure 13. These data provide one criterion for selection of "standard" dogs. Although for the most part the weight gains have been reasonably consistent, those for some animals have fallen well outside normal bounds. Such dogs are not ordinarily placed on main-line experiments.

General Survey of Experiments

To date, 73 dogs have been placed on experiments involving irradiation (Table 15). Except in Experiments 001 and 002, these animals were all 13 months $\pm$ 2 weeks of age at injection.

TABLE 15

Summary of radiation experiments

Expt. No.	Description	No. of dogs
001,003	Toxicity of Ce^{144} , intravenous	24
006	Studies of Ce^{144} distribution and excretion at low doses, intravenous	6
002, 004	Toxicity of Cs^{137} , intravenous	15
007	Effects of Cs^{137} in pregnant dogs	2
005	$\text{LD}_{50/30}$ of Co^{60} γ -rays	17
Controls		9
Total		73

A colony of control animals is being established by placing every 10th dog weaned into the control status. These animals serve as control material for all experiments. In addition, some control data are obtained from each dog up to the date it enters an experiment.

Figure 12. Weights of all untreated, outbred male beagle dogs from birth through 13 months of age

Figure 13. Weights of all untreated, outbred, virgin female beagle dogs from birth through 13 months of age

The developments in these experiments within the last six months are summarized below:

Toxicity and Metabolism of Ce¹⁴⁴

Experiment 003

Thirteen-month-old dogs were injected intravenously with a solution containing Ce¹⁴⁴ as the citrate. The doses and the survival are shown in Table 16. The animals were divided into three groups given, respectively, doses of 100, 50 and 25 $\mu\text{c}/\text{kg}$; 115 $\mu\text{c}/\text{kg}$ proved to be very close to the LD_{50/30}. There have been no additional deaths in the 50- and 25- $\mu\text{c}/\text{kg}$ groups since the two at 50 $\mu\text{c}/\text{kg}$, noted in the previous semiannual report.

Table 16

The toxicity of Ce¹⁴⁴ in 13-month-old beagle dogs

Experiment 003			
Dog number	Ce ¹⁴⁴ dose, μc	Dose, $\mu\text{c}/\text{kg}$	Survival after injection, days
0001	1136	126	26
0002	1041	107	31
0003	871	101	55
0007	468	54	>355
0009	491	54	>355
0011	487	55	>355
0012	427	51	>355
0013	427	52	>355
0022	437	47	78
0030	465	47	90
0014	240	28	>341
0015	216	28	>341
0016	246	28	>341
0017	281	28	>341
0020	287	27	>341
0021	281	29	>341
0026	194	24	>313
0031	198	25	>313
0032	243	25	>313
0033	176	23	>313

At this time the 50- and 25- $\mu\text{c}/\text{kg}$ dogs have survived almost one year since injection. They are in apparent good health. However, their hemograms show significant depressions in numbers of cellular elements. The body weights, especially in the 50 $\mu\text{c}/\text{kg}$ group, also show some evidence of a deviation from normal (Figs. 14 and 15).

Figure 14. Weights of 7 dogs injected intravenously with $50 \mu\text{c Ce}^{144}/\text{kg}$

Figure 15. Weights of 10 dogs injected intravenously with $25 \mu\text{c Ce}^{144}/\text{kg}$

It has become increasingly apparent - through radioautography of tissues and by α -counting techniques - that the Ce^{144} preparations supplied by Oak Ridge contain appreciable quantities of an α -emitting radionuclide, probably Pu^{239} . Preliminary estimations show a substantial fraction of 1% of the total radioactivity to be due to this contaminant. This is sufficient to contribute heavily to the clinical effects produced by injection of Ce^{144} at doses around or below $25 \mu\text{c}/\text{kg}$. This complication is now being examined in more detail.

Distribution and Excretion of Ce^{144} Injected Intravenously in Low Doses

Experiment 006

Six 13-month-old beagles were given Ce^{144} citrate intravenously. Three were each given a total of $7.5 \mu\text{c}$ Ce^{144} ; the remaining three were each given a total of $14.0 \mu\text{c}$ Ce^{144} . These differences in dose level are of no significance and relate to the fact that the injections were performed on separate occasions. The purpose of the study was to determine whether the distribution and rate of excretion of Ce^{144} was independent of dose level. With the low doses used here it is doubtful that any radiation-induced effects could be measured.

The dogs were measured at intervals for total Ce^{144} retention using whole-body scintillation counting techniques. They are being sacrificed at regular intervals and the Ce^{144} distribution among the various organs is being determined.

The data are still incomplete, but a preliminary view indicates certain differences in Ce^{144} distribution as compared to that in the high-dose dogs of Experiment 003. The most conspicuous difference in distribution is in the spleen, which contains a much smaller fraction of the injected dose in the low-dose dogs. The data will be presented in more detail in the next semiannual report.

Toxicity and Metabolism of Cs^{137}

Experiment 002

Cs^{137} was given intravenously to 5 adult "Michigan" beagles (cf. Reference 1). Two dogs given about $2000 \mu\text{c}/\text{kg}$ died at 27 and 64 days, respectively, after injection. However, the others, one of which was given $1500 \mu\text{c}/\text{kg}$ and the two other about $1000 \mu\text{c}/\text{kg}$, survived (Table 17).

In these surviving dogs, the white cell counts fell appreciably with minimal values around 30 days after injection. Thereafter their condition improved, and eventually the white counts stabilized, but at values appreciably lower than before injection. These dogs were then reinjected, 8 to 10 months after the first injection, with about the same doses given

previously. The actual doses are also given in Table 17. The postinjection loss in white cells was repeated about as described above, with recovery following again.

TABLE 17
The acute toxicity of Cs^{137} in beagles

Experiment 002					
Dog No.	Date 1st injection	Dose, μc	Date 2nd injection	Dose, μc	Dose level (each dose), $\mu\text{c/kg}$
0124	2-16-61	19,761	10-2-61	16,600	~ 1500
0114	10-31-60	7,802	8-4-61	11,000	~ 1000
0128	10-31-60	11,750	8-4-61	12,400	~ 1000

Experiment 004			
Dog number	Cs^{137} injected, μc	Dose level, $\mu\text{c/kg}$	Survival after injection, days
0054	17,050	1932	> 265
0055	17,170	2107	> 265
0081	15,520	1854	> 265
0246	22,517	2430	> 104
0247	21,861	2160	> 104
0256	18,504	2270	> 104
0257	20,202	2230	> 104
0258	24,657	2320	> 104

These dogs are still alive; their hemograms will be followed, and they will be injected a third time when and if their blood picture is again stabilized. The weights of two of these dogs are plotted in Fig. 16; these show no significant changes due to the injections of Cs^{137} .

Figure 16. Weights of dogs 0114 and 0128 which were each given two doses of about $1000 \mu\text{c/kg}$ of Cs^{137} . The first dose was given at time zero, the second 278 days later.

Experiment 004

As stated in our last progress report,⁽¹⁾ 13-month-old Argonne beagles were given intravenous doses of Cs^{137} at levels of approximately 2000 $\mu\text{c}/\text{kg}$. In spite of dramatic postinjection reductions in the numbers of formed blood elements, all of these dogs have survived, and their hemograms have shown considerable recovery.

Subsequently, in another group of five 13-month-old dogs, the dose of Cs^{137} was increased to about 2500 $\mu\text{c}/\text{kg}$. The picture noted above was substantially repeated. All these animals have survived so far (see Table 17) over a period of more than 3 months since injection.

It is apparent that the $\text{LD}_{50/30}$ for Cs^{137} in 13-month-old beagles is well in excess of 2000 $\mu\text{c}/\text{kg}$. Plans are now being made to use still higher doses to approximate the $\text{LD}_{50/30}$ for Cs^{137} .

The Effects of Cs^{137} Injected into Pregnant Bitches

Experiment 007.

Two 3-year-old females (0103 and 0039) were exsanguinated via the heart after receiving an anesthetic dose of sodium pentothal on the 24th and 25th days after intravenous injection with Cs^{137} . The dose of Cs^{137} , 250 $\mu\text{c}/\text{kg}$, was relatively low, perhaps 1/10 or less the $\text{LD}_{50/30}$ (see Exp. 004). In the instance of one (0039) that was injected approximately 30 days after conception, euthanasia was performed 55 days after conception, or 5 days before the expected delivery date.

Macroscopic findings included petechiae in the mucosa of the hard palate in one (0103), and in the thymus gland, tonsils, kidneys and vagina in the other (0039).

The marrow in the long bones of both animals was reddish-purple in the head, and fatty throughout the shaft and distal ends. This was similar to findings in unirradiated animals of similar age.

The liver of one (0039) contained several 1-mm cream-colored foci on the surfaces of all lobes. Similar lesions appeared on the surface of the renal cortex.

Microscopic observations in the non-pregnant animal (0103) included necrotic debris within lymphoid follicles, numerous eosinophils and many hemosiderocytes in the lymph nodes and spleen. In the latter, megakaryocytes were numerous.

Within the liver there were scattered small areas of hepatic cells as well as individual ones that were necrotic. Acidophilic intranuclear crystalloids were present within hepatic cells.

Calcium deposits occurred within the collecting tubules of the kidney.

The ovary contained three corpora lutea and several Graafian follicles. The uterus was in the anestrus stage.

Microscopic findings in the pregnant animal (0039) included multifocal areas of degeneration and necrosis together with cell infiltrations in the skeletal muscles, hyaline degeneration and necrosis in the myocardium.

Nucleated red blood cells were scattered throughout the hepatic sinusoids. The Kupffer cells contained large amounts of blood pigment, necrotic debris and erythrocytes (as many as six were seen per cell). Focal areas of round cell infiltration were distributed throughout the lobules and periportal spaces of the liver. Many binucleated hepatic cells were found. Intranuclear acidophilic crystalloids were seen within hepatic and Kupffer cells. There were numerous megakaryocytes in the spleen.

Foci of round cell infiltration (lymphocytes, plasma cells, and macrophages) appeared in the renal cortex near the capsular surface.

No attempt was made to culture the tissues for bacteria. Microscopic examination of sections prepared from heart, striated muscle, liver and kidney stained for Gram positive and negative bacteria failed to disclose a bacterial agent.

Of the eight fetuses removed shortly after the death of the dam, only two, a male and a female, were used for histologic study (0355 and 0357). These two had never breathed. On macroscopic examination, they were found to be well-developed, normal, nearly full-term fetuses. Their total body weights were small compared with those of healthy newborn puppies, which weigh 300 g or more. The weights of these two (0355 and 0357) were 243 and 236, respectively.

Microscopic study disclosed the following in both fetuses: focal myocarditis with hyaline degeneration and necrosis and diffuse fatty change of the liver parenchyma.

The lesions of striated muscle seen in the dam (0039) and two of her fetuses may not be directly associated with the administration of Cs¹³⁷. Similar lesions have been described in association with migrating parasites and viral agents. Future studies of similar animals will reveal whether this lesion is frequent or rare following Cs¹³⁷ administration.

The Lethal Action of Co^{60} γ -Radiation in Dogs

Experiment 005

Two objectives were in evidence when this experiment was started. Most conspicuous was the question whether radiation damage derived from a radionuclide such as Cs^{137} is actually a special case of uniform whole-body irradiation - special in the sense that the dose rate changes slowly over many days at a rate determined by the excretion of the isotope. The maximum permissible concentrations for Cs^{137} are estimated on the assumption that this is true. However, measurements of Cs^{137} concentrations in the tissues of dogs⁽¹⁾ show wide variations from uniformity - by factors of at least 5 to 10.

The second reason was to obtain a basis for subsequent checks to determine whether radiosensitivity in this dog population will remain relatively constant over a long period of time. Although radiosensitivity has not been observed to vary markedly in most mammalian species, data from experiments with monkeys and guinea pigs have shown appreciable variation - depending presumably on the state of health of the subjects.

Such observations may lead to a basis for more accurately comparing the effects of external, whole-body irradiation with those from certain internal emitters.

Because large numbers of dogs could not be devoted to this study, an estimate of the $\text{LD}_{50/30}$ for whole-body γ -irradiation was taken from the literature. All animals were given this dose, 325 r, measured in air. Seventeen dogs in all were irradiated at different times in groups of 5, 4, and 8, respectively. The first group of 5 was inadvertently irradiated unilaterally and all survived. The remainder were irradiated bilaterally. Air dose measurements, using ionization chambers, were supplemented by glass-rod microdosimeters taped to both sides of the dogs. The 325-r dose was given in a period of 30 min. The survival times after irradiation are given in Table 18. Of the 12 bilaterally irradiated dogs 8 survived and 4 died. In viewing the survival times of the two bilaterally irradiated groups there is a strong suggestion either that (a) there were some undetected differences in dosimetry or (b) the two groups of dogs did not respond uniformly. However, in either case, it is apparent that the $\text{LD}_{50/30}$ is close to 325 r. This corresponds to an absorbed dose of about 250 rads.

Of the four animals dying within 17 to 24 days following bilateral exposure (0245, 0251, 0252, and 0192), all exhibited generalized hemorrhages in all tissues on macroscopic examination. The thoracic lesions were most severe. Hemorrhagic pleural and pericardial effusions, hemorrhagic extravasations within the mediastinum, diaphragm, intercostal muscles, lungs, heart and pericardium were associated with fibrous and fibrinous adhesions

among the thoracic organs and wall. Pulmonary edema and blood-tinged, frothy fluid within the trachea were the terminal events.

TABLE 18

Survival of dogs given 325 r of Co γ -radiation

Dog No.	Date irradiated	Type	Survival, days
0147	7-25-61	Unilateral	>160
0148	"	"	>160
0149	"	"	>160
0150	"	"	76*
0151	"	"	>160
0245	8-22-61	Bilateral	17
0250	"	"	>132
0251	"	"	24
0252	"	"	23
0176	10-4-61	Bilateral	> 89
0177	"	"	> 89
0178	"	"	> 89
0179	"	"	> 89
0188	"	"	> 89
0189	"	"	> 89
0191	"	"	> 89
0192	"	"	19

*Sacrificed

The gastrointestinal tract was markedly altered by hemorrhagic change. A large hemorrhagic extravasation occurred in the esophagus at its junction with the stomach. There were several large hemorrhages in the serosa of the esophagus at the point where it touches the base of the heart. The mucosa of the stomach was studded with petechial and ecchymotic hemorrhages. At the junction of the duodenum with the stomach, the former contained mucosal hemorrhagic effusions. There were a few additional hemorrhages, though small, throughout the mucosa and serosa of the small intestine. Hemorrhagic effusions discolored the mucosa and serosa of the rectum. Ulcerated areas occurred in the mucosa. In one animal (0245) a perforated ulcer was noted in the rectum.

The kidneys contained numerous capsular and cortical hemorrhages. Most of the hemorrhages in the cortex were near the capsular surface. A few small hemorrhages appeared in the mucosa and serosa of the urinary bladder.

Several large hemorrhages were noted on the surface and cut section of the testicular, epididymal and prostatic glands.

The tonsils were enlarged, pitted and dull green to cream colored. Each was surrounded by a diffuse hemorrhagic area. The lymph nodes and spleen were homogeneously dark reddish-purple on surface and cut section. The thymus glands were markedly discolored by numerous large hemorrhages.

The surface of the liver in one (0252) was pitted with cream-colored lesions; on sectioning the organ, numerous 1-mm foci similar to those noted on the surface were seen throughout the parenchyma. The mucosa and serosa of the gall bladder were hemorrhagic.

The marrow of the long bones was reddish-purple throughout the cavity. The marrow within the shaft varied from fatty to gelatinous.

No hemorrhages were seen within the uterus and ovaries of the one female (0192).

Microscopic examination of the hematopoietic tissues revealed lymphoid and bone marrow aplasia. The lymphoid organs were distended with erythrocytes, eosinophilic granular albuminous material and fibrin. No vestige of a lymphoid follicle could be found. A few plasma-like cells and numerous macrophages heavily laden with blood pigment and reticulo-endothelial cells were apparently the only cells present in addition to massive numbers of erythrocytes.

No evidence of myelopoiesis could be found in the bone marrow cavity. The sinusoids were occupied with erythrocytes, phagocytic cells and plasma-like cells.

Bacteria were found within free and fixed reticulo-endothelial cells in liver, lymph nodes, bone marrow, kidney and adrenal. In one dog (0252) bacteria were also found in the tissue surrounding necrotic foci in the liver, kidney, pancreas and adrenal gland.

Focal areas of necrosis occurred in association with hemorrhage in the heart, adrenal cortex, cerebrum, kidney and tonsil; ulcerated areas occurred in the tonsils and the gastrointestinal tract.

The alveoli of the lungs were filled with eosinophilic granular albuminous material and erythrocytes. The alveolar capillaries were distended with red blood cells.

Within the liver, individual necrotic hepatic cells were seen. Occasional hepatic cell nuclei contained acidophilic crystalloids similar to those described previously.⁽¹⁾ The Kupffer cells contained necrotic debris and blood pigment in addition to bacteria.

Acidophilic granular material partly filled the Bowman's space. The glomerular tuft epithelium was diffusely vacuolated.

The adrenal cortex was completely or nearly devoid of lipids. The thyroid glands contained non-granular areas wherein the acinar epithelium was necrotic or absent, leaving colloid appearing in small irregular islands. The cytoplasm of the beta cells in the islets of Langerhans was vacuolated.

One dog (0150), sacrificed 76 days after unilateral exposure, was exsanguinated via the heart after receiving an anesthetic dose of sodium pentothal. Macroscopic alterations noted in this animal were localized in the bone marrow. That of the right humerus and femur was fatty throughout the shafts, whereas that of the left was reddish-purple throughout.

On microscopic examination the marrow within the left humerus and femur was hyperplastic; although all series of blood-forming cells were present, eosinophilic granulopoiesis predominated. The marrow of the right humerus and femur contained elements of the erythropoietic and granulopoietic series in the ratio of 2:1.

The lymphoid tissues were hyperplastic with the exception of the spleen which was hypoplastic. All lymphoid tissues contained massive numbers of eosinophils.

Marked adrenal cortical hyperplasia occurred in the glands, the ratio of cortex to medulla being nearly 3:1.

Urine and Blood Chemistry. Dogs 0147 and 0151, which were unilaterally irradiated, were placed in metabolism cages for collection of urine. Blood samples were also taken at intervals. Both materials were analyzed for glucose. Blood glucose values remained very close to the normal value of 75 mg percent at all times after irradiation. However, a reducing substance appeared in the urine of both dogs at 2-3 days after irradiation. Calculated as glucose measured by reduction of Benedict's solution, the concentration of this substance was about 0.5%. This substance decreased slowly with time after irradiation, the half-time being about 25 days, and was no longer detectable after 160 days.

This reducing substance was not glucose, however, and efforts to identify it are presently in progress.

Reference

1. Norris, W. P., L. S. Lombard, C. E. Rehfeld, and T. S. Speckman. Progress report: Toxicity and metabolism of radionuclides in dogs. I. Semiannual Report of Biological and Medical Research Division, Argonne National Laboratory. ANL-6464 (1961), pp. 50-71.

EFFECT OF NITROGEN MUSTARD ON THE RADIORESTORATIVE COMPONENT OF AMOEBA PROTOPLASM

Edward W. Daniels

In the course of a continuing attempt to identify the radiorestorative component in unirradiated amoeba protoplasm, it became of interest to determine whether or not this component could be rendered ineffective by nitrogen mustard. In this experiment, lethally X-irradiated giant amoebae received, by microtransfer, portions of protoplasm from other amoebae previously treated with nitrogen mustard.

Amoebae of the radiosensitive species Pelomyxa illinoisensis were placed for 1 hr in a solution containing 0.1 mg methyl-bis-(β -chloroethyl)-amine hydrochloride (Mustargen) per ml. This was a lethal dose and all treated controls died, but the amoebae lived for a number of days after treatment, thus allowing time for micromanipulative procedures. Another group of similar amoebae were exposed to X-rays at twice the lethal dose, that is, to 30 kr. This exposure killed all of 110 irradiated control animals after approximately 4 days. Neither X-irradiated nor mustard-treated control amoebae divided prior to death, but some of the latter lived longer.

The experimental group consisted of 30 of the X-irradiated individuals, each of which received, by microtransfer, a portion of a mustard-treated donor cell. These transfers consisted of 10 to 25% of the donor cell and included both cytoplasm and nuclei. Following the transfer, each amoeba was placed in a separate container and cultured. Of these amoebae, 70% underwent mitosis, and most of the dividing cells produced mass cultures within a month. Nearly all of the experimental organisms that did not divide lived longer than the X-irradiated controls.

Similar results were obtained in later experiments in which Mustargen at a higher concentration, 2 mg/ml, was used to treat the donor amoebae. It is clear from this work that the damage caused by X-radiation in these amoebae differs from that produced by the radiomimetic drug.

RECOVERY OF REPRODUCTIVE FUNCTION IN SUPRALETHALLY X-IRRADIATED AMOEBAE FOLLOWING CYTOPLASMIC MICROTRANSFER

Edward W. Daniels

Radiosensitive giant amoebae (Pelomyxa illinoensis) were exposed to 30 kr of X-rays, more than twice the lethal dose. This dose was sufficient to produce death and complete cellular disintegration in 400 X-irradiated control amoebae within 8 days, with no evidence of cell division. About 40 X-irradiated amoebae were given unirradiated cytoplasm by microtransfer methods within 2 days after exposure.

The cytoplasm for microtransfer was obtained from unirradiated amoebae that had been first centrifuged for 10 min at 700 x g, placed in ice water to preserve stratification, and then cut into halves. Nuclei, crystals, food vacuoles, some mitochondria, and a small amount of cytoplasm were concentrated in the centrifugal or heavy halves; the light halves contained vesicles of various types, lipid globules, Golgi structures, some mitochondria, and a large amount of groundplasm, throughout which are scattered electron-dense granules about 350 A in diameter. The heavy halves were discarded in this experiment although they possess radio-restorative capability, and the centripetal or light halves were collected for individual examination for nuclei. Each light half of an amoeba was put into a newly designed micrurgical chamber and flattened sufficiently for observation of the nuclei in a phase microscope. Occasional light halves were found that contained one or more nuclei. These nuclei were removed by a micropipette or the entire fraction with nuclei was discarded; only the contents of completely enucleated light halves were transferred into supralethally X-irradiated recipient amoebae. Following microtransfer, each amoeba was isolated and cultured.

The survival of the treated group was 84% at 10 days after X-ray exposure; the last of the X-irradiated untreated control had disintegrated two days before. Of the amoebae that survived 10 days, 81% produced mass cultures within a relatively short time. For comparative purposes, 40 unirradiated amoebae were isolated directly from stock cultures and observed as controls; after 10 days, 88% were alive, and nearly all of them gave rise to mass cultures within a second 10-day period.

These data show that nuclei of unirradiated cells are not required for survival and reproduction of supralethally irradiated amoebae, at least if some components of unirradiated cytoplasm are provided. Further efforts are being made to isolate and describe the essential radiorestorative components in donor systems.

PROGRESS REPORT: EXPERIMENTAL STUDIES ON THE
Cu(I,II)-PEROXY THEORY OF RADIOBIOLOGICAL
EFFECTS AND THEIR MODIFICATION

Biological Studies

Joan F. Fried and Jack Schubert

The effect of sodium cyanide on radiation sensitivity has been explored further in rats and mice. This compound has the ability to stabilize cuprous ion, Cu(I), by means of complex formation.⁽¹⁾ Assuming that at least one effect of radiation on mammalian tissue is the inhibition of cytochrome c oxidase activity by interference with Cu(I)-Cu(II) transformations, one might expect that stabilization of Cu(I) by means of complex formation with cyanide could alter the radiation sensitivity of the organism. From a consideration of the law of mass action, it was thought possible that this effect could vary from sensitization to protection, depending on the dose of cyanide employed.

Mature Sprague-Dawley (SD) female rats and CF No. 1 female mice, of equal weight and age, were selected for irradiation at two doses of X-irradiation each. The dose rates used were 20 r/min for rats and 38 r/min for mice. Sodium cyanide was administered intraperitoneally 3 to 5 min prior to the irradiation, at doses ranging from 0.5 mg/kg to the minimum lethal dose for the species. There were 20 to 35 animals in each irradiated group at each dose level of cyanide. Percent survival, weights and time to 50% death were recorded.

A preliminary toxicity study on NaCN, administered by the intraperitoneal route, was performed. The data (Table 19) indicate that the LD_{50/30} of NaCN is about 6.5 mg/kg for mice and about 4.0 mg/kg for rats.

TABLE 19

Acute toxicity of intraperitoneally injected
NaCN in mice and rats

Species	No. injected	NaCN, mg/kg	% Survival
Mouse*	9	5	100
	12	6	75
	6	7	0
Rat*	6	3	100
	6	4	50

*The mice were CF No. 1 females; the rats were Sprague-Dawley females.

The effect of NaCN on the radiation sensitivity of rats was explored at 800 and at 850 r. Figure 17 shows that at 850 r, the dose of NaCN necessary to achieve maximum protection was in the minimum lethal range (3.5 mg/kg); at 800 r, the over-all protection was considerably less, but was reached at a lower dose of NaCN (survival of rats treated with 2.5 mg NaCN per kg was 37%; that of the controls was 20%).

Figure 17. The effect of NaCN on the survival of SD female rats, irradiated at 800 and 850 r. Each point represents data from at least 20 animals (except for 1.0 mg/kg at 850 r, where 10 were used).

Mice were irradiated at 650 and at 750 r (Figure 18). At 750 r, the maximum protective dose of NaCN was not reached below a level of 5.5 mg/kg, whereas at 650 r, maximum protection was present over a dose range of from 3.0 to 5.5 mg/kg.

Figure 18. The effect of NaCN on the survival of CF No. 1 female mice, irradiated at 650 and 750 r. Each point represents data from 20 or 30 animals, (except at 5.5 and 6.0 mg/kg of NaCN at 750 r, where 9 and 6 were used, respectively).

These results are of interest from two standpoints:

1. They demonstrate that some protection is afforded the rat by NaCN. The literature reveals some conflicting data with regard to this point. Dowdy et al.⁽²⁾ observed no protection using a dose of NaCN slightly below the minimum toxic level. On the other hand, Yakovlev and Ivanov,⁽³⁾ using NaCN in the minimum lethal range as we have done here, obtained a small protective effect (survival of treated, 33%; controls, 0%).

2. Yakovlev and Ivanov suggest that the minimum protective dose of NaCN is directly proportional to the radiation dose, i.e., as the radiation dose is increased the minimum protective dose of NaCN is also increased. Since the mechanism employed to increase the X-ray dose is to prolong exposure time rather than to increase the dose rate, it is possible that some of this effect may be due to increased detoxification of the CN^- by the liver during the irradiation process. However, since the increase in exposure time is small (5% to raise the dose from 800 to 850 r in rats and 15% to raise the dose from 650 to 750 r in mice), it is unlikely that it is the only factor.

Little or no tendency towards sensitization is demonstrated at any of the doses of cyanide employed.

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PROGRESS REPORT: EXPERIMENTAL STUDIES ON THE
Cu(I,II)-PEROXY THEORY OF RADIOBIOLOGICAL
EFFECTS AND THEIR MODIFICATION

Biochemical Studies

Jack Schubert and William M. Westfall

Our last report⁽¹⁾ described a radiation-induced inactivation of the oxygen-combining ability of hemocyanin. Further experiments in this series have now been carried out to define the chemical species responsible for this oxidative inactivation in γ -irradiated hemocyanin systems.

Catalase was used to identify and separate any effect produced by hydrogen peroxide from effects produced by other oxidizing molecules resulting from irradiation. Ninety micrograms of a high purity catalase preparation were added to 2 6.0-ml samples of *Limulus* hemolymph (1.067×10^{-3} M in Cu) before irradiation; 2 identical samples were reserved for addition of catalase after irradiation, and 2 samples without added catalase were reserved for controls. All solutions were irradiated at 1000 r/min with Co^{60} γ -rays to a total accumulated dose of 60,000 r. Catalase was added to the 2 postirradiation catalase samples. All solutions were allowed to stand at 22°C for $\frac{1}{2}$ hr to allow sufficient time for decomposition of H_2O_2 by catalase, and all solutions were then deaerated for 2 hr using oxygen-free, water-saturated helium.

All solutions were then reaerated using a stream of water-saturated air, and 1:20 dilutions in phosphate buffer were subjected to optical density (OD) measurements at 340 m μ . It has been shown by Redfield and others⁽²⁾ that the OD of hemocyanin at 340 m μ is directly proportional to its oxygen content. The OD of a solution containing 15 μg catalase per ml is negligible: <0.002 OD at 340 m μ . The percentage decrease in OD at 340 m μ was computed using the OD of unirradiated, He-air-cycled hemolymph as representing 100% oxygenation.

The results are given in Table 20. Catalase present during irradiation abolishes the effect of irradiation on the O_2 -combining capacity of hemocyanin. After irradiation catalase abolishes 80% of this effect. We assume that the residual 20% which cannot be destroyed by catalase is due to organic peroxides which are known to be produced by ionizing irradiation and to be resistant to the attack of catalase.⁽³⁻⁵⁾

Similar experiments with similar results have been carried out at a hemocyanin concentration 5% of the above.

TABLE 20

Effect of catalase on the radiation-induced inactivation of oxygen-combining ability of hemocyanin

Treatment		% decrease in OD at 340 m μ
Irradiation, r	Catalase	
None	None added	0.0
60,000	None added	A 13.7
		B 10.8
60,000	Added before irradiation	A 0.8*
		B 0.6*
60,000	Added after irradiation	A 2.3
		B 2.3

*Percentage increase in OD over that of unirradiated controls.

As a further check on the identity of oxidizing agents produced by irradiation of hemolymph, peroxide assays were carried out on irradiated hemolymph. The triiodide method⁽⁶⁾ was used to determine "active" peroxide. This particular method assays both hydrogen peroxide and the more reactive organic peroxides (i.e., per-acids, low molecular weight epoxides and similar compounds). Limulus hemolymph was diluted 1:20 with 0.05 M phosphate buffer (pH 7) and irradiated with 40,000 r of Co⁶⁰ γ -rays. The hemolymph was not deaerated (which would destroy any peroxides present by allowing them to combine with deoxyhemocyanin) but was stored at 4°C. At intervals, aliquots of this solution were ultrafiltered through Visking casings to remove hemocyanin, which absorbs light at the same wavelength as I₃⁻, and the peroxide content was determined.

As shown by Ghiretti,⁽⁷⁾ hemocyanin has a catalase-like action in decomposing H₂O₂. The "catalase" activity of hemocyanin is very low on a mole protein basis but accounts for the peroxide decomposition as measured here (Table 21). Organic peroxides are known to decompose spontaneously in aqueous solutions even at low temperatures,⁽⁵⁾ and this effect probably accounts for the very small amount of peroxide detected at 120 hr.

When the hemolymph solutions described above were deaerated at the same time intervals their O₂-combining ability decreased in proportion to the amount of peroxide remaining. A sample deaerated at 0.4 hr after irradiation lost 88% of its O₂-combining ability. At 48 hr only 47% of the O₂-combining ability was lost on deaeration.

TABLE 21

Peroxide decomposition in irradiated
Limulus hemolymph

Time after irradiation, hr	Peroxide (as H ₂ O ₂), μM/liter
.75	49.1
24	17.2
48	13.2
120	1.6

Thus a direct relationship has been shown between peroxide production and loss of O₂-combining ability of hemocyanin in our system. The peroxides produced are hydrogen peroxide and a substance, or substances presumed to be organic peroxides.

A hemolymph sample has been subjected to standard methods of blood analysis and at least one constituent, free amino acid, appears to be present in sufficient concentration to be a source of organic peroxide. Okada⁽⁸⁾ has shown that some free amino acids produce amino acid peroxides with a G value (molecules amino acid peroxidized/100 ev) of about 1. The best source of radiation-produced amino acid peroxides appears to be those amino acids with a tertiary carbon atom (i.e., valine and leucine). Limulus hemolymph has an amino acid nitrogen content of 2.4 mg percent, corresponding to a 1.14×10^{-3} M amino acid concentration (assuming a molecular weight of 144 and an N content of 6.9%).

Further studies are in progress to determine the source of organic peroxides in this system, and to clarify dose-response relations.

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THE SIMULTANEOUS DETERMINATION OF C^{14} AND H^3 IN BIOLOGICAL MATERIAL, USING THE SCHÖNIGER COMBUSTION TECHNIQUE

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For the determination of C^{14} and H^3 , as well as other nuclear species in combination, there is an increasing interest in the Schöniger rapid combustion technique.⁽¹⁻³⁾ This technique for the most part eliminates quenching problems, and biological samples up to 300 mg can be handled conveniently.

This article presents a method for the simultaneous determination of C^{14} and H^3 using nonylamine in ethanol as the absorbing solvent. Nonylamine was chosen rather than hyamine because of its higher scintillating efficiency in this system.

Material and Methods

The instrument used in this study was a Packard Tri-Carb liquid scintillation spectrometer equipped with discriminators to select certain channels. The other materials needed are gelatin capsules (weighing about 100 mg), a 2-liter Schöniger combustion flask provided with a 30-ml separatory funnel that allows the addition of liquids to the flask without opening it; a platinum ignition-electrode system, surrounded by a basket of platinum gauze, with the inserts attached in a rubber stopper; a 0-6 volt transformer with an ignition push-button; and the standard Wheaton 15-ml liquid scintillation counting vials.

The sample is weighed out in the gelatin capsule. A nick is cut in the capsule in such a way that it is possible to attach it to the electrode. The electrode system is placed in the combustion flask, and the flask is filled with oxygen, closed, and placed behind a safety screen. The sample is then ignited and combustion is allowed to proceed. The transformer is set so that the ignition-electrode becomes light red a half-second after the ignition push-button is pushed. After combustion has been completed, the flask is placed with its bottom in a dry ice-chloroform bath to freeze out the water. After one-half hour the flask is removed from the cooling mixture, 20 ml of 0.5 M nonylamine in ethanol (100%) is slowly added. Five minutes later 25 ml of scintillation solution (2,5-diphenyloxazole in toluene) is added. From time to time the flask is shaken to insure complete mixing.

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After 2 hr the flask is opened, and three 12-ml aliquots are taken for assay. An H^3 -standard is added to one of the aliquots and a C^{14} -standard to another. All three samples are then counted in the liquid scintillation spectrometer at a temperature of $5^\circ C$. All three aliquots are counted in two channels. Channel 1 requires discriminator settings of 20- ∞ v and a high voltage of 940 v (tap 5); channel 2 requires discriminator settings of 5-20 v and a high voltage of 1400 v (tap 11).

Experimental Results and Calculations

Table 22 shows the results obtained from various samples to which were added known amounts of H^3 , C^{14} , or both, in the presence of a representative biological tissue, in this case, powdered lyophilized rat liver. The absolute activity of the H^3 and C^{14} was previously determined by gas-phase counting using a glass proportional counter.⁽⁴⁾ H^3 - and C^{14} -labeled toluene were used as internal standards. In most cases each figure represents the mean of at least 6 determinations. The recoveries obtained by the rapid combustion technique indicate that the method is essentially quantitative, and correction for quenching is made directly in the calculation of the activities.

TABLE 22

Activities obtained by adding known amounts of H^3 , C^{14} , or both to representative biological materials

Composition of mixtures*			Calculated activity		Recovery	
C^{14} -benzoic acid (15.9×10^{-5} $\mu c/mg$), mg	H^3 -liver, (10.9×10^{-5} $\mu c/mg$), mg	Liver, (no activity), mg	C^{14} -benzoic acid, 10^{-5} $\mu c/mg$	H^3 -liver, 10^{-5} $\mu c/mg$	C^{14} , %	H^3 , %
15.3		52.4	17 ± 2	3 ± 4	107	
18.9		51.3	17 ± 2	2 ± 4	107	
	41.7		$0 \pm 1^*$	107 ± 4		98
	111.9		$0 \pm 1^*$	110 ± 4		101
14.0	97.5		16 ± 2	109 ± 4	101	100
15.2	100.9		17 ± 2	108 ± 4	104	99

*Values calculated for 15 mg benzoic acid.

For calculating the activity of H^3 and C^{14} in each others' presence under these conditions of measurement the following calculations are used:

Let CC = activity of C^{14} in sample
 CH = activity of H^3 in sample
 SC = activity of C^{14} due to internal standard
 SH = activity of H^3 due to internal standard
 E_C^1 = efficiency of C^{14} in channel 1

E_H^1 = efficiency of H^3 in channel 1

E_C^2 = efficiency of C^{14} in channel 2

and E_H^2 = efficiency of H^3 in channel 2

In the aliquot without an internal standard the count rate is as follows:

$$\text{Channel 1: } C_C E_C^1 + C_H + E_H^1 = a$$

$$\text{Channel 2: } C_C E_C^2 + C_H + E_H^2 = b$$

In the aliquot with the internal C^{14} standard the count rate is:

$$\text{Channel 1: } (C_C + S_C) E_C^1 + C_H E_H^1 = c$$

$$\text{Channel 2: } (C_C + S_C) E_C^2 + C_H E_H^2 = d$$

In the aliquot with the internal H^3 standard the count rate is:

$$\text{Channel 1: } C_C E_C^1 + (C_H + S_H) E_H^1 = e$$

$$\text{Channel 2: } C_C E_C^2 + (C_H + S_H) E_H^2 = f$$

The final activity can then be calculated as follows:

$$C_H = \frac{S_H [b(c-a) - a(d-b)]}{(f-b)(c-a) - (e-a)(d-b)}$$

and

$$C_C = \frac{S_C [a(f-b) - b(e-a)]}{(f-b)(c-a) - (e-a)(d-b)}$$

It is possible to derive similar equations for the situation in which one aliquot contains a combined internal standard, C^{14} plus H^3 , in contrast to the one used here, in which two aliquots contain C^{14} and H^3 , respectively.

Discussion

The method described has been successfully applied to a mixture of C^{14} - and H^3 -labeled materials. One may wonder how such a low statistical error can be obtained using the equations presented here. This is the result both of the relative high activity of the internal standard solutions used and of the choice of channels and voltage settings. With other instruments slightly different voltage and discriminator settings may be more convenient.

In our situation, channel 1 usually gives no counts due to H^3 ; accordingly the factor (e-a) is almost always very small. Other observations of quenching phenomena indicate that in some biological materials counting is associated with a considerable shift of the H^3 spectrum. The presented method of calculation automatically corrects for these unpredictable quenching effects.

It is possible to avoid the counting of three aliquots, i.e., to do all the counting on only one sample. In this case the sequence is; count, add C^{14} standard, count, add H^3 standard and count again. In this case it is recommended that the activity of the H^3 standard be 100 times larger than the activity of the C^{14} standard. This sequence leads, however, to a larger statistical error, because the counter settings with respect to voltage are not exactly reproducible with the time.

It should be mentioned also that although these calculations were used specifically for C^{14} and H^3 , they can be used for mixtures of other isotopes such as Ca^{45} and P^{32} .

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SILICA GEL STRUCTURE AND THE CHROMATOGRAPHIC PROCESS: SURFACE ENERGY AND ACTIVATION PROCEDURES

Peter D. Klein

A previous study in this series⁽¹⁾ dealt with the structure of the adsorbent and its influence on the adsorption and differential migration of sterol acetates. The data presented there as well as in the current work pertain especially to the sterols, but the findings also appear to be applicable to chromatographic separations in general since they focus attention on the role of the structure of the adsorbent.

The present report deals with the surface energy of the adsorbent and the roles of activation and deactivation in the achievement of optimum column performance. In previous studies of this subject, the structures of the silica gels used were either not reported⁽²⁻⁴⁾ or were exclusively of the high-surface-area, small-pore type.⁽⁵⁾ This study deals with three silica gels of different known structures in columns in which cholesterol acetate was the migrating compound. The effectiveness of the gel treatment was measured by the ratio of the retention volume to the bandwidth of the adsorbate. This criterion was selected because of its direct applicability to the prediction of separation efficiencies.

Theoretical

The surface of a silica gel does not possess a uniform energy; an adsorbate molecule will be held more tightly in one region of the surface than in another, owing to the differences in the types of chemical groups on the surface and in the local geometry of neighboring particles of the gel. Three, perhaps four, types of chemical groups can be distinguished. The first, the silanol ($-\text{Si}-\text{OH}$) group, predominates in the usual commercial gel. During gelation and subsequent drying of the gel, some of these groups undergo dehydration to form siloxane ($-\text{Si}-\text{O}-\text{Si}-$) linkages between neighboring silicon atoms. Other silanol groups persist in a third form, as the hydrated silanol group ($-\text{Si}-\text{OH}-\text{O}\overset{\text{H}}{\underset{\text{H}}{\text{H}}}$), even after drying. It is possible also that some siloxane linkages may still continue to hold a water molecule by physical adsorption, although this possibility is extremely unlikely at ambient relative humidities less than 50%. Rehydration of siloxane linkages to silanol groups does not occur at an appreciable rate in atmospheres below saturation with water vapor.

Each of these three groups possesses a different average energy; for example, Brunauer *et al.*⁽⁶⁾ reported that the energy of the siloxane surface in amorphous silica is 259 ± 3 ergs/cm² (as determined by the

heats of solution in nitric-hydrofluoric acid mixtures), whereas the silanol surface was calculated to have an energy of 129 ± 8 ergs/cm². These widely separated values ostensibly support the belief that physically adsorbed water can be completely removed from the surface before chemisorbed water is removed by the dehydration of silanol groups. However, the range of energies present was such that Brunauer et al. were forced to assume that "...the average heat of adsorption of water physically adsorbed on the most active fraction of the silanol surface was approximately equal to the average heat of hydration of the entire siloxane surface." (original italics).

The reasons for this wide range in energy values may well lie in the local geometry of the surface. The silica gel surface is not a plane crystalline surface but an amorphous one made up of particles, predominantly spheroidal, in an agglomerate mass. The orderliness of the surface exists only in local patches, if at all, and is influenced by the particle radius. In addition, regions between particles may exist where the physically adsorbed water molecule or the prospective adsorbate molecule is acted upon by two neighboring surfaces at the same time, with a resultant increase in the adsorptive energy.⁽⁷⁾

The conventional silica gel adsorbent has a surface energy and an associated energy distribution range that reflect the structure of the gel and its past history. The experimental approach to these considerations must therefore be a dual one, the first concerned with the identification of surface conditions which exist in the gel, the second with the chromatographic behavior of adsorbates on such gels.

In the present study, a further subdivision of the first aspect was carried out in order to study the effects of hydration on the gel surface. Each of the gels was stored for three weeks at 98% humidity and room temperature in an attempt to "anneal" any areas of siloxane bonds which might have been produced by commercial drying procedures. Individual portions of these gels and their original counterparts were then equilibrated under a variety of conditions. Selected samples were ignited to obtain the combined physisorbed and chemisorbed (silanol) water contents. All samples, after equilibration, were tested in the standard chromatographic column. The performance was evaluated by means of the retention volume, d, required to elute 50% of the test sample, and by the bandwidth, w. These measurements were used previously⁽¹⁾ to calculate the numbers of "plates" generated in the column $[(4d/w)^2]$. In the present report the direct ratio, d/w, is also employed. Finally, the two aspects, surface chemistry and column performance, are correlated to rationalize chromatographic behavior in terms of the surface energy and body structure of the adsorbent.

Experimental

Silica gels. The three gels used in this study were products of the Davison Chemical Company, Baltimore, Md. and are referred to by the Davison type code as #12, MS and #62. These gels represent the small-pore, medium-pore and large-pore structure respectively; the particular lots employed in this study were characterized by nitrogen adsorption isotherms and other measurements reported in the first paper.⁽¹⁾ This characterization includes specific surface area, pore volume, average pore diameter and bulk density (Table 23).

TABLE 23
Silica gel properties

	#12	MS	#62
Specific surface area, m ² /g	805	547	274
Pore volume, cc/g	0.41	0.92	1.16
Average pore diameter, A	20.5	67.0	170.0
Bulk density, g/cc	0.61	0.40	0.29
Equivalent particle diameter, A*	34	50	101

*Calculated on the basis of spheres of density 2.20

Gel treatments. Each of the three gels was used in two conditions: as the original gel (OG) and after hydration by storage in an atmosphere of 98% humidity for three weeks (FH). Each of the gels was subjected to 10 treatments covering 3 types of deactivation or activation: storage in known humidities for 3 weeks, drying at elevated temperatures, and vacuum drying at known pressures (Table 24). Direct comparison of the two gel conditions was provided by the static equilibrations; the original gels were further activated by the thermal treatments while the fully hydrated gels were treated by vacuum drying. Thermal treatments were not applicable to the fully hydrated gels because of the pronounced sintering characteristics of such material.⁽⁸⁾

Ignition studies. Weighed samples of the treated silica gels were ignited in platinum crucibles at 1100°C for 4 hr or until constant weight was attained. Pilot studies of hydrated samples showed that pre-ignition heating over a bunsen flame was required to reduce "boiling" and loss of sample. The weight loss on ignition (considered to be all water) and the specific surface area were used to calculate the number of hydroxyl groups per square millimicron (p. 236 of Reference 9); this value was then converted to percent silanol coverage on the basis of Iler's value of 8 hydroxyl groups per square millimicron for amorphous silica (p. 242 of Reference 9).

TABLE 24
Gel treatments

Treatment Code	Static equilibration
S 1	3 weeks at 11.02% humidity (saturated LiCl solution)
S 2	3 weeks at 7.05% humidity (saturated NaOH solution)
S 3	3 weeks at 0.08% humidity (granulated CaCl ₂)
	Thermal treatment
T 1	18 hr at 50°C, 5 mm pressure
T 2	18 hr at 110°C, atmospheric pressure
T 3	18 hr at 150°C, atmospheric pressure
T 4	18 hr at 550°C, atmospheric pressure
	Vacuum drying
V 1	2 hr at 1×10^{-5} mm, room temperature
V 2	18 hr at 2×10^{-4} mm, room temperature
V 3	18 hr at 1×10^{-6} mm, room temperature

Operation of the chromatographic column. The apparatus and method of operation has been described previously.⁽¹⁾ The conditions employed throughout this study were as follows: gel mesh size, 200-325; sample load, 2.0 mg cholesterol acetate; eluting solvent, 16% benzene in hexane (v/v); flow rate, 15-20 ml/hr. Fractions were collected in the conventional manner; sterol concentrations were determined by the Liebermann-Burchard reaction. The graphical method described previously⁽¹⁾ was used to obtain the retention volume $\bar{d}$ and the bandwidth $\bar{w}$ of the adsorbate.

Results

Table 25 lists the percent water lost on ignition of the original and fully hydrated gels after a series of treatments. From these values are calculated the apparent percentages of the surface existing in the silanol configuration; these are listed as percent hydroxyl coverage.

TABLE 25
Water content and hydroxyl coverage of silica gels after various treatments

Treatment Code	#12				MS				#62			
	Original gel		Fully hydrated		Original gel		Fully hydrated		Original gel		Fully hydrated	
	%H ₂ O*	%OH**	%H ₂ O	%OH	%H ₂ O	%OH	%H ₂ O	%OH	%H ₂ O	%OH	%H ₂ O	%OH
S 1	14.14	141	14.62	145	10.93	155	9.10	129	4.18	126	4.48	135
S 2	12.02	120	13.10	131	8.92	127	9.62	128	3.85	116	4.13	124
V 2	-	-	8.45	84	-	-	5.68	80	-	-	3.29	99
S 3	7.26	73	8.02	80	5.66	80	5.51	78	2.64	80	4.11	123
V 3	5.99	60	-	-	4.75	68	-	-	2.63	79	-	-
T 4	1.98	20	-	-	1.98	28	-	-	0.92	28	-	-

*Equivalent to weight loss on ignition at 1100°C

**Apparent percent silanol surface.

The surfaces of gels that had been equilibrated over granulated calcium chloride (S3) (which probably represents the most moderate technique for removing physically adsorbed water) were 20-25% denuded of silanol groups, and except possibly for the large-pore gel (#62), they were not permanently restored to complete silanol coverage by the hydration treatment. Vacuum drying of the original gels at 10^{-6} mm for 18 hr (V3) also resulted in surfaces that were only partially covered by silanol groups. The influence of gel structure on the stability of silanol groups is seen in the comparison of the three gel surfaces after vacuum drying or after high temperature treatment (T4); the higher the original surface area, and consequently the smaller the particle radius, (Table 23) the greater the loss of silanol hydroxyl groups observed for a given treatment.

The losses induced by ignition in gels previously stored at 11% and 7% humidity (S1, S2) illustrate the potentially deceptive nature of these measurements, even when coupled with the nitrogen isotherm values for the basic gel structure. It is not possible, by single measurements of this type, to distinguish between a fully hydrated surface partially covered with physically adsorbed water and a partially dehydrated surface completely covered with water. In these experiments, the former situation is considered unlikely because of the persistence of siloxane surfaces in gels which had been stored under conditions designed to bring the surface to full hydration; that is, if denuded areas exist after storage at 98% humidity (S3), they would still exist at 11% humidity.

Table 26 lists the retention volumes of cholesterol acetate when eluted by 16% benzene in hexane from the gels in various stages of dehydration. The range of retention volumes achieved by these treatments decreased in order of surface area of the gels, being greatest with #12 (482 ml) and least with #62 (131 ml). The relative order of retention volumes produced by given treatments also differed from gel to gel. For example, drying at elevated temperatures was much less effective than vacuum drying in increasing the retention volumes in the large-pore gel. In part at least, this may be due to the lower bulk density of the large-pore gel which would retard the diffusion of water vapor in static thermal drying at atmospheric pressure.

TABLE 26
Retention volumes and plate numbers of cholesterol acetate on silica gels activated by different procedures

#12				MS				#62			
Ret. vol.	Plate numbers	Gel condition	Treatment code	Ret. vol.	Plate numbers	Gel condition	Treatment code	Ret. vol.	Plate numbers	Gel condition	Treatment code
71	123	FH	S 1	104	152	FH	S 1	67	268	OG	S 1
74	211	OG	S 1	120	334	OG	S 2	68	196	FH	S 1
93	200	FH	S 2	125	270	FH	S 2	70	123	FH	S 2
107	235	OG	S 2	126	192	OG	S 1	76	177	OG	S 2
157	108	OG	T 2	213	121	FH	S 3	106	75	OG	T 2
217	249	FH	V 1	228	334	OG	S 3	109	78	OG	T 2
317	198	FH	S 3	259	167	FH	T 1	111	119	OG	T 3
318	198	OG	S 3	276	275	FH	V 2	118	80	FH	V 1
367	144	FH	V 2	286	126	OG	T 1	133	104	FH	S 3
460	159	OG	T 3	317	165	FH	V 1	134	119	OG	S 3
553	123	FH	V 3	339	134	FH	V 3	154	79	FH	V 2
				374	76	OG	T 2	198	37	FH	V 3
				377	140	OG	T 3				

The static equilibrations (S-series) provide the best direct basis for the comparison of the original gels with their counterparts which had been previously stored at 98% humidity for three weeks. As stated, the objective of the hydration treatment had been to restore the siloxane groups to silanol groups, thus hopefully reducing the diversity both of the numbers of groups and of the surface energies associated with them. A comparison of the plate numbers generated in the column, however, showed a distinctly deleterious effect of prior hydration at high humidities.

Figure 19 shows a composite graph wherein the ratio d/w is plotted vs $\log d$, for each of the three gels. The points were fitted to a straight line equation by the least squares method, and the calculated slope together with the associated standard error is given for each gel.

Despite the variety of treatments and the attendant scatter of values, two features of the effect of activation are clearly visible; first, no gel showed any perceptible improvement in column performance (d/w) as retention volume was increased; second, there was instead a detrimental effect whose severity increased as the pore diameter of the gel increased.

Figure 19. The effect of activation procedures on column performance. Open circles, original gels; filled circles, fully hydrated gels.

Discussion

The data presented here clearly indicate the individuality of silica gel structures as judged by their responses to similar treatments and again emphasize the necessity of knowing the basic structural properties of the adsorbent used in a particular chromatographic procedure. Without this knowledge, it is impossible to compare or, indeed, reproduce observations concerning the chromatographic properties of the adsorbent. This is particularly true of silica gels from other manufacturers which are sold without statement or warranty of surface area and pore volume.

The evaluation of column performance, in the last analysis, has the practical objective of improving the separation of a series of compounds. For this reason, criteria that have been used in the past to judge the effectiveness of activation or deactivation, e.g., some fixed retention volume⁽²⁾ or symmetry of the elution peak⁽⁵⁾ have been replaced by the

ratio of the retention volume to the bandwidth. This simple relationship, together with the relative retention volumes for the absorbate compounds, leads directly to a prediction of the column length necessary for a given separation by a calculation analogous to that in gas-liquid chromatography.⁽¹⁰⁾ This criterion also has several other advantages. By focussing on the ratio d/w as a function of d , the smallest retention volume consistent with the maximum ratio can be selected, thus providing the conditions whereby the chromatogram is developed in minimum time and the peak concentration of the sample is maximal. The latter feature has more than trivial importance in separations involving limited amounts of biological material. The most important benefit of this criterion, however, is that it provides a convenient yardstick for judging the progressive effectiveness of activation or deactivation. In the plot of d/w vs $\log d$, three types of slope are possible: positive, indicating continuous improvement as activation continues; negative, implying a detrimental effect; and little or no slope, suggesting the indifferent situation in which the elution is delayed without effect on column efficiency. Figure 19 demonstrates not only that positive slopes are not likely to be encountered in silica gel activation, but that the response of the column performance will range from indifference to intractability depending on the gel structure.

Ideally, it would be desirable to have an adsorbent whose surface is characterized by a single type of functional group possessing a uniform energy; alternatively, the presence of several types of groups on the surface would not be objectionable if the energy distribution were fairly narrow. Achievement of the first objective is unlikely because of the extensive overlap between the energy required to remove physically adsorbed water molecules and that required to dehydrate any two silanol groups to a siloxane linkage. The experiments with gels stored in a fully hydrated condition further suggest that it is not feasible to rehydrate siloxane bonds to silanol groups and thereafter to remove physically adsorbed water to achieve a pure silanol surface. Gels treated in this manner and subsequently dried at room temperature either by equilibration over calcium chloride or in vacuo had only slightly higher silanol coverages than untreated gels. These results are evidence that elevated temperatures are not required for the dehydration of silanol groups, a finding already noted by Brunauer et al.⁽⁶⁾ who reported that a silica gel dried by equilibration at 25° at 0.5 μ contained only 82% of the bound water predicted from the nitrogen area for a pure silanol surface.

Since the attainment of a single type of chemical group on the surface of silica gels thus appears impossible, it may be asked: what treatment is likely to reduce the energy distribution of a mixed population? From thermodynamic considerations,⁽³⁾ it seems likely that any progressive increase in the average energy of the surface will lead to a greater range in the distribution of surface energies which, in turn, will translate

itself into a disproportionately broader and more asymmetric peak. On the other hand, deactivation procedures, if carried far enough, will result in one or both of two events: first, a reduction in surface energy to the point where the adsorbate molecule is no longer retained by the adsorbent; and second, physical obstruction of the porous structure to the extent that large portions of the adsorbent surface become unavailable to the adsorbate. Either of these events becomes the limiting condition in the improvement of adsorbent performance by deactivation procedures. Optimum conditioning of a given gel, therefore lies, slightly below the point where these two events take place. The optimum hydration is not, however, the same for all gel structures and must be determined on an individual basis. In this connection it is interesting to note that the large-pore structure of #62 permits a greater degree of deactivation than the other gels before the performance deteriorates. This may be attributable to the existence of large interparticle "crevices" as well as to the large openings interconnecting the pore structure.

Finally, the selection of deactivation procedures deserves some comment. Previous workers have used liquid water to prepare partially deactivated adsorbents, in weight proportions up to 24% of the gel weight.⁽⁵⁾ Aside from the obvious danger of misjudging the monolayer capacity of silica gels of different structures by this means, the additional variable of siloxane bond hydrolysis by liquid water is introduced. The comparison of gels that had been stored in a fully hydrated condition (though short of the vapor pressure at which liquid water surrounded the particles) with untreated gels showed that such treatment had a distinctly deleterious effect on column performance. For this reason, as well as those pertaining to uniformity and the attainment of true equilibrium, the use of controlled, known humidities for deactivation⁽¹¹⁾ is to be preferred.

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SOME EFFECTS OF IONIZING RADIATION ON METHIONINE SULFONIUM COMPOUNDS

Fritz Schlenk and Julia L. Dainko

Methionine sulfonium compounds are cellular constituents of plants, animals, and microorganisms.⁽¹⁾ Their biochemical functions include the role of methyl donors in transmethylation. Several reports⁽²⁻⁶⁾ on the sensitivity of methyl group metabolism to ionizing radiation suggested an exploration of the effects of X-rays and γ -rays on methionine sulfonium compounds; the latter are the source of methyl groups of numerous metabolites. While methionine has been investigated rather extensively in this respect,⁽⁷⁻⁹⁾ there appears to be no record of radiation studies of its sulfonium derivatives.

The present report deals with observations on the action of X-rays and γ -rays on S-methylmethionine and S-adenosylmethionine, and on the enzyme systems responsible for the biosynthesis of the latter compound in yeast.

Methods and Materials

Yeast and chemical compounds. Commercial preparations of *Saccharomyces cerevisiae* (active dry yeast) were used as a source of S-adenosylmethionine and for the study of its biosynthesis.^(10,11) In one instance *Candida utilis* ATCC 9950 was used because of its superior quality for ultraviolet photomicrography.⁽¹²⁾ S-Methylmethionine was obtained by synthesis.⁽¹³⁾ Purification and conversion of the sulfonium iodide to the sulfate was achieved by chromatography with Dowex 50 H^+ -resin, development with sulfuric acid, and isolation by means of phosphotungstic acid; the procedure was analogous to that described for S-adenosylmethionine.⁽¹⁰⁾ 5'-Methylthioadenosine was obtained by hydrolysis of S-adenosylmethionine,⁽¹¹⁾ and 3-methylmercaptopropylamine was prepared by thermal decomposition of methionine.⁽¹⁴⁾ All other compounds were of commercial origin.

Irradiation procedures. X-Irradiation of the compounds and cells was carried out at 25°C. Water or dilute phosphate buffer at or near neutrality was used as the solvent or suspending fluid, and some yeast samples were irradiated in the dry state ($5 \pm 1\%$ moisture). Gamma-ray-irradiations were carried out in the Argonne High Level Gamma-Irradiation Facility. Solutions of the samples were sealed in glass ampoules and kept frozen before and after irradiation.

Analytical procedures. The analytical techniques used for sulfonium compounds were those developed in earlier studies.⁽¹⁰⁾ Advantage was taken of the strongly cationic properties of these substances which facilitate chromatographic separation. The use of sulfonium compounds labeled in various positions further facilitated the quantitative analyses⁽¹⁵⁾ and the paper chromatographic identification of fragments produced by radiation.

Results

The sensitivity of solutions of S-adenosylmethionine toward X-irradiation was investigated. In high dilution, the effects resemble those observed with other adenine derivatives, as indicated by a decline of the optical density at 260 m μ .^(16,17) Two reasons for this appear possible: The double bonds of the adenine part of the molecule may suffer destruction, or adenine may be split from S-adenosylmethionine; the latter process would be attended by a drop of the optical density at 260 m μ from 15,400 to 13,100.⁽¹⁸⁾ Because of the low concentration of material used in the experiment, no chemical analysis was possible. Exposure to 20,000 r at a concentration of 10 μ M/ml proved inefficient; no significant changes in absorption spectrum were noted at this level, nor were decomposition products detected by paper chromatography. Experiments with S-methylmethionine at this concentration and dose level likewise remained inconclusive. Higher doses of X-irradiation were tested, therefore, and the results are given in Table 27. The compounds used were labeled in the methyl group to facilitate analysis after chromatographic separation of the intact sulfonium compound from its products of decomposition.⁽¹⁵⁾ The decline in optical density of S-adenosylmethionine solutions at 260 m μ was small compared with the destruction of the sulfonium group listed in Table 27; thus, the effect on the adenine part of the molecule and on the glycosidic linkage is rather limited.

TABLE 27

Decomposition of methionine sulfonium compounds by X-irradiation

Dose of X-irradiation, kr	% Decomposition of S-methylmethionine		% Decomposition of S-adenosylmethionine	
	7.8 μ M/ml	1.6 μ M/ml	3.6 μ M/ml	1.8 μ M/ml
100	4.8	11.7	3.0	5.6
200	9.4	24.3	5.4	10.1
300	14.5	36.6	8.4	17.6
400	18.1	43.2	14.5	21.4

The compounds were dissolved in water. The conditions of X-irradiation were, 250 kv, 30 ma, 3.12 mm Al, target distance 23 cm, 1020 r/min.

Similar experiments were carried out to test the stability of the methionine sulfonium compounds toward γ -irradiation (Table 28). Here, too, S-methylmethionine is more sensitive than S-adenosylmethionine. The extent of degradation of the sulfonium group after 4.0 megarads was sufficient to permit a paper chromatographic search for the fragments. The parent substances were labeled (S^{35} , methyl- C^{14} , and C^{14} in the α -position of the amino acid moiety). Nearly half of the activity of the methyl-labeled compounds was found in volatile fragments after irradiation with 4 megarads; methyl mercaptan and dimethylsulfide could be discerned easily by organoleptic tests with nonlabeled sulfonium compounds. Chromatography of the nonvolatile compounds revealed numerous substances, among which methionine, accruing from either sulfonium compounds, was prominent. Many of the products observed in this survey may be secondary derivatives of methionine, and, since the multifarious radiodecomposition of methionine has been studied extensively before,⁽⁷⁻⁹⁾ the present effort was restricted to a preliminary survey. Only a fraction of the paper chromatographic spots was identified. Among the fragments derived from both sulfonium compounds were methionine, methionine sulfoxide, 3-methylmercaptopyrrolamine, 3-aminopropanol, homoserine, and homoserine lactone. In addition to these, 5'-methylthioadenosine and its sulfoxide as well as adenine and hypoxanthine were obtained from S-adenosylmethionine.

TABLE 28

Destruction of the sulfonium group in methionine
sulfonium compounds by γ -irradiation

Total dose, megarad	Dose rate, rad/min	% Destruction of S-adenosylmethionine		% Destruction of S-methylmethionine	
		10 μ M/ml	2 μ M/ml	10 μ M/ml	2 μ M/ml
2.0	2.56×10^4	16.9	44.0	30.8	67.6
4.0	2.73×10^4	30.0	66.6	47.1	78.2

The stability of S-adenosylmethionine toward X-irradiation was investigated with the material located in the vacuoles of yeast cells.⁽¹⁹⁾ The results are indicated in Table 29. Since the vacuole occupies only a fraction of the cellular volume, the concentration of the sulfonium compound in this compartment must be very high. This may explain why the destruction by X-irradiation remained relatively low. Ultraviolet photomicrography of the irradiated cells indicated no change in morphology; S-adenosylmethionine had remained in the vacuoles. This is in accord with the spectrophotometric analyses of the suspending water (Table 29). The sulfonium compound was not released in significant quantities from the vacuoles of the yeast cells into the cytoplasm, nor through the cell membrane into the surrounding medium. Both membranes remained

retentive under the conditions of the experiment. This stability of internal organization of the yeast cells encouraged a survey of the radiation sensitivity of the enzymatic equipment.

TABLE 29

Analysis of X-irradiated yeast cells (*Candida utilis*)

X-ray dose, kr	Suspending medium*		Cell extract (1.5 N PCA [†])*	
	Nucleotide fraction, OD** x vol.	S-Adenosylmethionine, μ M	Nucleotide fraction, OD** x vol.	S-Adenosylmethionine, μ M
0	2	0.3	286	11.1
50	2	0.4	278	10.4
100	2	0.5	283	10.7
200	2.4	0.6	314	11.7
400	3.0	0.4	316	10.0

*A 10% suspension of freshly harvested cells enriched with S-adenosylmethionine was exposed to X-irradiation: 250 kv, 30 ma, 3.12 mm Al, target distance 23 cm, 1020 r/min. Suspending fluid and cells were analyzed separately by ion exchange chromatography. All data are derived from 1.0 g moist packed cells.

**Optical density.

[†]Perchloric acid.

The resistance of the enzymes involved in the production of S-adenosylmethionine was studied by exposure of dried and hydrated preparations of *S. cerevisiae* to X-irradiation (Table 30). After exposure, each sample was introduced into 100 ml of culture medium supplemented with 75 mg of L-methionine. After incubation for 24 hr at 30°C with vigorous aeration, the cells were washed and analyzed via perchloric acid extraction and chromatography.⁽¹⁰⁾ The yield of cells was found rather uniform, and little gain in weight beyond hydration had occurred. Regardless of the dose of X-irradiation, the amount of S-adenosylmethionine was found constant within each series in four experiments with two different yeasts. In two instances, one involving a superannuated yeast sample, the yield after exposure to 400 kr was only 70 and 75% compared with the unirradiated controls. The time course of formation of the sulfonium compound was virtually identical in both instances and very similar to the data reported earlier⁽¹¹⁾ for unirradiated yeast. Plating of the irradiated cells at zero time and after the 24-hr period of metabolism showed no significant change in colony formation, and repetition of the experiment on sulfonium compound biosynthesis with a yeast sample 4 and 16 days after the irradiation gave the same results with respect to lack of viability of the cells, yet unimpaired ability to synthesize S-adenosylmethionine from methionine, ammonium ion, and glucose.

TABLE 30

Formation of S-adenosylmethionine in X-irradiated yeast (*S. cerevisiae*)

X-irradiation, kr	Plate count of survivors, %	Recovery of yeast after metabolism, g/100 ml	S-Adenosylmethionine in PCA [†] extract, $\mu\text{M/g}$
0	100	3.1	0.3*
0	100	3.1	29.8
100	10.2	3.1	31.8
200	0.4	3.2	31.4
300	0.02	3.3	31.2
400	0.002**	3.3	31.4
0	100	2.6	0.7*
0	100	2.6	26.2
100	1	2.6	26.8
200	-	2.7	27.1
300	-	2.7	26.5
400	-	2.8	26.1

X-Irradiation: 250 kv, 30 ma; 3.12 mm Al; T.D. 23 cm; 1020 r/min.

Upper Series: Dried yeast cells were irradiated; in the lower series a 10% suspension of cells in 0.1 M phosphate, pH 6, was irradiated.

*This experiment was carried out in the absence of the methionine supplement in the medium.

**After 24 hours in the culture medium the number of viable cells had increased to 0.01%.

[†]Perchloric acid.

These experiments were extended to γ -irradiation (Table 31). With doses as high as 1.0 megarad, little decline occurred in the capacity to produce the sulfonium compound. The effect at higher doses may be explained by damage to the cell membranes as well as to the enzymes. Much organic matter was found in the incubation medium, and microscopic examination revealed deterioration of the cells.

TABLE 31

Biosynthesis of S-adenosylmethionine by yeast after γ -irradiation

Radiation dose, megarad	S-Adenosylmethionine formed, $\mu\text{M/g}$	
	Yeast A	Yeast B
0	20.4	20.7
0.5	21.9	21.7
1.0	19.5	18.0
2.0	4.1	2.4
4.0	0.3	0.3

The yeast was irradiated in the dry state in sealed ampoules. Samples of 0.75 g were introduced into 100 ml of medium containing 5.0 μM L-methionine per ml. Harvest of the cells, extraction, and chromatographic analysis were carried out after 40 hr incubation at 30°C.

Discussion

The present experiments show that S-adenosylmethionine and S-methylmethionine are not unusually sensitive toward X- and γ -irradiation; they resemble the parent compound methionine in this respect. The sulfonium group is decomposed somewhat faster than other parts of the molecule and, in contrast to the response toward acid and alkali,^(18,20) S-methylmethionine is more sensitive toward ionizing radiation than S-adenosylmethionine.

The relatively advanced state of knowledge concerning the localization⁽¹⁹⁾ and biosynthesis⁽¹⁰⁾ of S-adenosylmethionine in yeast cells has now permitted a survey of the effects of ionizing radiation on this system. After lethal doses of X-rays, the yeast cell releases only minute quantities of S-adenosylmethionine through the vacuolar and cytoplasmic membranes into the suspending medium. Ultraviolet spectrophotometry of the latter indicates that the retentiveness of the cell is preserved for nucleotides and related substances as well.

The enzymatic equipment of the yeast cells for production of S-adenosylmethionine was found very resistant toward X- and γ -irradiation. After 400 kr of X-irradiation in the dry as well as in the hydrated state, the cells showed in several instances undiminished ability to form the sulfonium compound, if sufficient L-methionine was provided in the medium. After γ -irradiation of yeast in the dry state, nearly full activity was observed after exposure to 1.0 megarad, while a sharp decline occurred after 2.0 megarads exposure.

For an appreciation of the extent of enzyme resistance, the metabolic conditions of the yeast should be recalled. The major reaction sequences leading to S-adenosylmethionine under the present conditions are as follows:

- (1) Glucose + NH_4^+ $\rightarrow$ Inosinic acid
- (2) Glucose $\rightarrow$ Ribose
- (3) Precursors $\rightarrow$ L-Methionine (rate-limiting)
- (4) Inosinic acid $\rightarrow$ Adenosinetriphosphate
- (5) Adenosinetriphosphate + L-methionine $\rightarrow$ S-Adenosylmethionine

Without supplemental L-methionine in the medium, the concentration of S-adenosylmethionine in the cells remains low ($< 0.5 \mu\text{M/g}$) regardless of irradiation. Addition of methionine and related sulfur amino acids increases the level up to 60-fold. The reaction sequences 1, 2, 4, and 5 are operative, therefore, at a much higher rate than in the absence of methionine supplement, and radiation damage to the enzymes involved should be more readily discernible.

The present data show that S-adenosylmethionine formation can take place in yeast cells that have been irradiated beyond viability in terms of colony formation. After exposure to one megarad of γ -irradiation the rate-limiting reaction of the sequence of steps involved (see equations 1 to 5) begins to show impairment, or perhaps another enzyme is damaged sufficiently to become rate-restricting. One may not conclude that all other enzymes concerned with the formation of S-adenosylmethionine in yeast are fully resistant under the conditions specified. There may be sufficient reserves of them to compensate for partial loss, but the conclusion seems warranted that none of the numerous enzymes is unusually sensitive. The present survey of cellular radiation sensitivity in yeast, while directed toward S-adenosylmethionine metabolism, provides data on the biosynthesis of several other important metabolites as well. Thus, the purine ring system, ribose, and ATP have many other functions besides being incorporated into S-adenosylmethionine. Their synthesis occurs unimpeded after the radiation doses specified although the cells in the presence of methionine have to make much larger amounts than usual.⁽¹²⁾ It appears certain from these data that radiation damage to the nucleic acid metabolism of yeast cells does not involve the purine-forming enzyme system.

Unfortunately, reliable studies of radiation effects on the biosynthesis of S-adenosylmethionine in liver are not possible, at present. The concentration in this tissue is low, and extraneous methionine does not stimulate the biosynthesis as much as in yeast cells.⁽²⁰⁾ A similar survey of the biosynthesis of S-methylmethionine must await more detailed knowledge of the mechanisms and enzymes involved.

Summary

S-Adenosylmethionine and S-methylmethionine show no unusual sensitivity toward X- and γ -irradiation. They resemble methionine and adenosine in this respect. S-Adenosylmethionine, located after biosynthesis in the vacuoles of yeast cells, is not released by 400 kr of X-irradiation. The enzymatic synthesis of the sulfonium compound including formation of the purine and ribose structure from simple precursors was virtually unimpeded after exposure of the yeast cells to 400 kr of X-irradiation or 1.0 megarad of γ -irradiation.

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PROGRESS REPORT: THE BIOSYNTHESIS OF METHIONINE

Utilization of S-Adenosylmethionine by Microorganisms

Stanley K. Shapiro

The function of S-adenosylmethionine as an important methyl donor has been clearly established in a variety of transmethylation reactions.⁽¹⁾ However, the relationship of this sulfonium compound to methionine biosynthesis is far less clear. In order to obtain information concerning the metabolic fate of S-adenosylmethionine as well as the demethylated product S-adenosylhomocysteine it seemed worth while to test the ability of various microorganisms to utilize these compounds and related compounds for growth.

Materials and Methods

Methods of culture. Escherichia coli (K 12) and A. aerogenes (NRRL 8308) were used as stock cultures. Mutants were produced by ultraviolet irradiation. The "complete" medium used in these experiments consisted of 0.5% Difco tryptone, 0.5% Difco yeast extract and 0.5% glucose. The "minimal" medium had the following composition per liter: K_2HPO_4 , 14 g; KH_2PO_4 , 4 g; sodium citrate, 0.5 g; $MgSO_4$, 0.1 g; $(NH_4)_2 SO_4$, 1.0 g; glucose, 5.0 g. For the preparation of a "sulfur-free" medium $MgSO_4$ and $(NH_4)_2 SO_4$ were replaced by equimolar amounts of $MgCl_2$ and NH_4Cl respectively.

Enzyme preparations. Cell-free extracts were prepared in a 10kc Raytheon sonic oscillator. Homocysteine-transmethylase activity was determined according to the tracer method of Shapiro and Yphantis.⁽²⁾ The methods used to detect the enzymatic decomposition of S-adenosylmethionine have been described previously by Shapiro and Mather.⁽³⁾

Results

A summary of the growth responses of the three major types of mutants is given in Table 32. Culture 62 is typical of the most common type of methionine mutant in that it is incapable of substituting S-adenosylmethionine for methionine. This is true of all methionine mutants that were tested except for the one special class of A. aerogenes mutants as exemplified by culture 68. As can be seen from the data presented this organism grows well on methionine, S-adenosylmethionine, 5'-methylthioadenosine or the combination of S-methylmethionine and homocysteine.

TABLE 32

Growth responses of mutants of A. aerogenes to various supplements

Supplement (0.2 μ M/ml)	Optical density at 650 m μ		
	#62	#68	AM-1
None	0.00	0.00	0.00
S-Adenosyl-L-methionine	0.02	0.57	0.53
L-Methionine	0.68	0.68	0.00
Adenine or adenosine	0.01	0.00	0.00
L-Methionine + adenine or adenosine	0.64	0.65	0.00
Methylthioadenosine	0.00	0.45	0.15
L-Homoserine	0.00	0.01	0.01
Methylthioadenosine + homoserine	0.00	0.46	0.16
L-Homocysteine	0.02	0.25	0.00
S-Methyl-L-methionine	0.01	0.00	0.01
Homocysteine + S-methyl-L-methionine	0.64	0.63	0.00
S-Adenosyl-L-homocysteine	0.01	0.02	0.00
S-Adenosyl-L-homocysteine + formate or serine	0.02	0.02	0.01

Growth responses were measured after 24 hr incubation on a rotary shaker at 37°C. "Minimal" medium was used with the supplements indicated.

Homocysteine alone permits only one-third to one-half maximal growth. Raising the concentration of homocysteine to 0.4 μ M/ml causes inhibition of growth. Mutant AM-1 is of course not a methionine mutant. The only compound that permits good growth is S-adenosylmethionine. It should be pointed out that many other compounds and combinations of compounds were used that yielded negative results. It is of interest to note that none of the cultures responds to S-adenosylhomocysteine alone or with one-carbon donors such as formate or serine.

Since α -amino- γ -butyrolactone has been shown to be the first enzymatic decomposition product of S-adenosylmethionine⁽²⁾ this compound was substituted for homoserine but was as inactive as homoserine in promoting growth of these mutants.

The only effective substitute for S-adenosylmethionine is supporting growth of culture AM-1 is a mixture of adenine and ribosylmethionine. In Table 33 the growth responses of mutant AM-1 are given in detail. It may be seen that while adenine or ribosylmethionine alone are inactive, yet the use of a mixture of both compounds results in growth responses closely parallel to these obtained with S-adenosylmethionine at equimolar concentrations.

TABLE 33
Growth responses of mutant AM-1

Concentration, μ M/ml	Optical density at 650 m μ				
	S-Adenosylmethionine	Adenine	S-Ribosylmethionine	Adenine + S-ribosylmethionine	5'-Methylthioadenosine
0.01	0.08	0.00	0.01	0.06	0.00
0.05	0.34	0.01	0.00	0.30	0.03
0.1	0.50	0.03	0.02	0.45	0.08
0.2	0.58	0.02	0.00	0.56	0.15
0.4	0.56	0.00	0.03	0.57	0.20
0.8	0.59	0.01	0.01	0.56	0.25

Experimental conditions as described for Table 32.

In either case the optimum responses is obtained at a concentration of 0.2 μ M/ml. The only other compound that permits detectable growth is 5'-methylthioadenosine. It may be seen that at the highest concentration used (0.8 μ M/ml) this compound supports up to one-half maximal growth, whereas at the concentration which supports maximal growth with S-adenosylmethionine, 5'-methylthioadenosine supports very limited growth. Homoserine or α -amino- γ -butyrolactone added to 5'-methylthioadenosine did not result in any increase in growth of the cultures.

The data in Table 34 show that mutants 68 and AM-1 as well as the wild type culture are able to utilize S-adenosylmethionine or 5'-methylthioadenosine as the sole source of sulfur. The wild type was included so as to demonstrate that during the time of the experiment the cells were depleted of sulfur reserves to the point where growth was negligible in the absence of an organic sulfur supplement. Cultures 62, 68 and the wild type also utilize methionine as a sole source of sulfur.

TABLE 34

Growth responses of mutants of *A. aerogenes* with various compounds as sole source of sulfur

Culture	Optical density at 650 m μ			
	Unsupplemented	Methionine	S-Adenosylmethionine	5'-Methylthioadenosine
<i>A. aerogenes</i>	0.05	0.65	0.68	0.64
#62	0.01	0.52	0.01	0.02
#68	0.00	0.68	0.56	0.44
AM-1	0.01	0.02	0.52	0.12

Growth responses were measured after 24-hr incubation on a rotary shaker at 37°C. The "sulfur-free" medium was used supplemented with 0.2 μ M/ml of the compounds listed above.

Cell-free extracts of different mutants were prepared and tested for enzymatic activity with S-adenosylmethionine as substrate. All extracts showed good activity in the transmethylation of homocysteine (Table 35).

TABLE 35

A comparison of the activity of the homocysteine transmethylases of three different mutants of A. aerogenes

Source of enzyme	Methionine formed, $\mu\text{M}/\text{mg protein/hr}$	
	S-Methyl(C^{14}H_3)-L-methionine	S-Adenosyl(C^{14}H_3)-L-methionine
<u>A. aerogenes</u>	1.0	0.3
#62	0.9	0.2
#68	1.1	0.4
AM-1	2.0	0.3

The reaction mixtures contained $8\mu\text{M}$ L-homocysteine and $8\mu\text{M}$ of the methyl donor per ml in 0.1 M phosphate, pH 6.4 and were incubated at 35°C . S-Methyl(C^{14}H_3)-L-methionine was used at a specific activity of $400,000\text{ cpm}/\mu\text{M}$, and methionine was determined on the basis of a specific activity of $200,000\text{ cpm}/\mu\text{M}$. S-Adenosyl(C^{14}H_3)-L-methionine was used at a specific activity of $425,000\text{ cpm}/\mu\text{M}$, and the resultant methionine had the same specific activity as the methyl donor. In all cases the results were calculated after subtraction of the control values obtained without L-homocysteine.

It may be seen that the homocysteine transmethylase of culture AM-1 was twice as active as any of the other transmethylases when S-methylmethionine was the methyl donor. With S-adenosylmethionine as the methyl donor all extracts showed similar activity. No other methyl donor has been found that replaces the two methionine sulfonium compounds. S-Adenosylhomocysteine was totally inactive in replacing homocysteine as the methyl acceptor.

In all cases when S-adenosylmethionine was used as the methyl donor, there was considerable enzymatic destruction of the sulfonium compound in the presence or absence of homocysteine. A comparison was made of this enzymatic degradation and the results are summarized in Table 36. Here again culture AM-1 shows approximately twice the activity of the other cultures as measured by the amount of S-adenosylmethionine that is decomposed. The three reaction products, adenine, methylthioribose and homoserine account for the decomposed S-adenosylmethionine in all cases. As shown in an earlier report⁽²⁾ the four-carbon fragment is actually α -amino- γ -butyrolactone which is nonenzymatically rapidly converted to homoserine. All four extracts decomposed $9.0\mu\text{M}$ of 5'-methylthioadenosine to $9.0\mu\text{M}$ adenine and methylthioribose under the same conditions as given in Table 36.

Since adenine and S-ribosylmethionine supported growth of culture AM-1 as well as S-adenosylmethionine, it seemed possible that the former two compounds were being used to synthesize S-adenosylmethionine. It was not possible, however, to demonstrate this latter reaction with cell-free extracts of AM-1 or any of the other mutants.

TABLE 36

The enzymatic decomposition of S-adenosylmethionine

Source of enzyme	Substrate	Reaction products, $\mu\text{M}/2\text{ml}/3\text{hrs}$				
		S-Adenosylmethionine	Methylthioadenosine	Adenine	Methylthioribose	Homoserine
<u>A. aerogenes</u>	S-Adenosylmethionine	7.2	0.1	1.8	1.7	1.9
#62	"	7.6	0.1	1.3	1.4	1.2
#68	"	7.0	0.1	1.9	1.8	1.8
AM-1	"	5.6	0.0	3.5	3.2	3.3

The reaction mixtures (2.0 ml) contained 10 mg protein of each extract, $9\mu\text{M}$ of substrate in 0.1 M phosphate, pH 6.8, and were incubated for 3 hr at 35°C .

Discussion and Summary

It seems clear that the ability to satisfy the methionine requirement of various microorganisms with S-adenosylmethionine is uncommon. This is in conformity with the fact that among the various enzymatic products of the degradation of S-adenosylmethionine, methionine itself has not been detected. The only methionine mutant that utilizes S-adenosylmethionine is culture 68 which also utilizes 5'-methylthioadenosine for growth. Cell-free extracts of this organism decompose S-adenosylmethionine readily to 5'-methylthioadenosine although in vitro the latter compound does not accumulate since it is decomposed as rapidly as it is formed. In vivo, however, it is likely that 5'-methylthioadenosine is the compound which enables the organism to grow. The manner in which this compound enters the pathway of methionine biosynthesis is unclear, although it clearly does since S-adenosylmethionine or 5'-methylthioadenosine may serve as the sole source of sulfur.

The other type of methionine mutant (#62) also contains the same enzymes that decompose S-adenosylmethionine, but presumably the genetic block in methionine biosynthesis is beyond the point where 5'-methylthioadenosine can enter the pathway. This is also suggested by the inability of these cells to utilize S-adenosylmethionine or 5'-methylthioadenosine as the sole source of sulfur.

Mutant AM-1 serves to demonstrate that S-adenosylmethionine is required for other metabolic purposes than as a devious source of part of the methionine molecule although the sulfonium compound may serve as the sole source of sulfur for the biosynthesis of methionine. This organism, apparently, has a genetic block that has nothing to do with methionine biosynthesis. The utilization of adenine and S-ribosylmethionine for growth suggests a new pathway for the biosynthesis of S-adenosylmethionine, but this lacks experimental evidence as yet.

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PROGRESS REPORT: PROPIONIC ACID METABOLISM

III. THE CONVERSION OF ACRYLATE TO SUCCINATE

Robert W. Swick and Isabelle S. Waldron

The mechanism of the isomerization of methylmalonyl coenzyme A (CoA) to succinyl CoA remains unresolved. The nature of this novel biochemical reaction is currently the subject of intensive investigation in a number of laboratories. Eggerer and associates⁽¹⁾ have proposed a rearrangement that involves a free radical intermediate similar to that suggested by Urry and Kharasch for the rearrangement of the phenylneopentyl radical.⁽²⁾ In accord with this hypothesis, Phares et al.⁽³⁾ recently reported an inhibition of the reaction by chlorpromazine, an excellent free-radical scavenger.

On the other hand, Kosower⁽⁴⁾ has suggested that the reaction involves a dehydrogenation which weakens the bond of the formyl CoA group, permitting its translocation to the other side of the double bond:

Since the existence of formyl CoA has never been demonstrated, it is assumed that the thioester would have to be bound to the enzyme and that perhaps free acrylic acid might then occur as a transient intermediate. This hypothesis has been tested by carrying out the isomerization of methylmalonyl CoA in the presence of isotopic acrylic acid and examining the succinate formed for radioactivity. Acrylate was indeed incorporated into succinate, however, not via the isomerization of methylmalonyl CoA. Further investigation revealed that acrylyl CoA was formed in the system, reduced to propionyl CoA and then converted to succinyl CoA by transcarboxylation and isomerization. The reduction of acrylyl CoA has not previously been demonstrated in the propionibacteria.

Methods

Acrylic acid labeled with C¹⁴ was prepared from β-alanine-3-C¹⁴ by deamination to β-hydroxypropionic acid and dehydration of the latter compound. Acrylic acid was purified by partition chromatography on Celite and stored at -10°C as a dilute solution in phosphate buffer.

Succinate was isolated by partition chromatography. Acrylic and propionic acids migrate together when the stationary phase is 0.2 N H₂SO₄ but may be separated satisfactorily when the acid is replaced by 1.5 M KH₂PO₄.

A cell-free extract was prepared by sonic rupture of either fresh or lyophilized cells of P. shermanii, centrifuged and either used directly or treated first with ammonium sulfate. Acrylate-C¹⁴ was incubated with these extracts in the presence of various unlabeled intermediates and the products formed were isolated and measured in a gas-flow counter.

Results

The simplest procedure was to incubate acrylate-C¹⁴ with the dialyzed extract and the intermediates of the transcarboxylation-isomerization system: propionate, CoA, ATP and oxalacetate. Under these conditions, succinate-C¹⁴ was readily formed (Table 37). However, treatment of the extracts with ammonium sulfate to precipitate the protein fraction resulted in the total loss of activity toward acrylate, while in control incubations propionate-C¹⁴ was still converted to succinate (Table 37). Furthermore, incubation of these otherwise active extracts with methylmalonyl CoA and acrylate-C¹⁴ led to no incorporation of radioactivity into succinate. Hence one may conclude that the incorporation of acrylate observed with the crude extracts was not occurring by way of the suggested mechanism, nor, apparently, is free acrylate an intermediate in the methylmalonyl isomerase reaction. Previous experiments have shown indirectly that propionate is not an intermediate.⁽⁵⁾ Barker⁽⁶⁾ recently reported his failure to achieve incorporation of acrylate, glycine, or propionate into β -methyl aspartate during the isomerization of glutamate, an analogous reaction.^(1,7)

TABLE 37

Formation of succinate from propionate-C¹⁴ and acrylate-C¹⁴

Additions	Succinate formed from 1 μ M of	
	Propionate-C ¹⁴ , μ M	Acrylate-C ¹⁴ , μ M
Crude extract + oxalacetate + CoA + ATP	0.580	0.330
Treated extract + oxalacetate + CoA + ATP	0.560	0.009
Treated extract + methylmalonyl CoA	0.100	0.001

However, it was of interest to investigate the conversion of acrylate to succinate by these extracts. Its incorporation was inhibited by avidin and was dependent on oxalacetate. The reduced activity observed when the extracts were dialyzed, and the complete disappearance of activity on treatment with ammonium sulfate, suggested that some cofactor was being removed. There is ample evidence that acrylyl CoA can be reduced to propionyl CoA in a number of systems, but previous attempts to demonstrate the reaction in the propionibacteria were negative. When either TPNH or DPNH was added along with CoA, ATP and oxalacetate, reduction of acrylate to propionate and subsequent conversion to succinate occurred (Table 38).

TABLE 38

Effect of TPNH or DPNH on conversion
of acrylate to succinate

Additions	Succinate formed from 1 μ M of acrylate- C^{14} , μ M
None	0.02
TPNH (5 μ M)	0.16
DPNH (5 μ M)	0.44

System contained: oxalacetate, CoA, ATP.

The reduction of acrylate to propionate was shown directly by the isolation of labeled propionate when acrylate- C^{14} was incubated in the same system minus oxalacetate (Table 39). Absence of CoA resulted in no reduction; therefore, the reaction appears to involve the CoA derivative, as has been shown to be the case in other systems.

TABLE 39

Formation of propionate from acrylate

Additions	Propionate formed from 1 μ M acrylate- C^{14} , μ M
CoA + ATP + DPNH	0.30
No DPNH	0.00
No CoA	0.00

The reaction could also be followed by observing the disappearance of the ultraviolet absorption at 340 $m\mu$ resulting from the oxidation of reduced pyridine nucleotide in the presence of acrylyl CoA. Here only DPNH

was active; TPNH was inactive (Table 40) in contrast to the earlier isotope experiments. Nor was there any transhydrogenase activity apparent in our extracts (Table 40). The isotopic evidence for the participation of TPNH in the reaction is perhaps an artefact. The enzyme involved is most likely propionyl dehydrogenase since it exhibits much less activity toward crotonyl CoA (Table 40). The enzyme also displays much less, although significant, activity toward acrylyl pantetheine. We are currently attempting to purify and characterize the enzyme.

TABLE 40

Specificity of propionyl CoA dehydrogenase

Additions	Pyridine nucleotide oxidized,* μM
Acrylyl CoA + DPNH	0.109
Acrylyl CoA + TPNH	0.000
Acrylyl CoA + TPNH + DPN	0.000
Acrylyl CoA + DPNH	0.199
Crotonyl CoA + DPNH	0.051

*Initial value: $0.4 \mu\text{M}$

The enzyme for the conversion of acrylic acid to acrylyl CoA appears to be present in our extracts, but the activity is not as high as that with propionate (Table 41).

TABLE 41

Conversion of acrylic acid to acrylyl CoA

Compound activated	Hydroxamate formed with $5 \mu\text{M}$ ATP, μM
Propionate	4.61
Acrylate	0.93
Propionate + acrylate	3.12

It was of interest to determine if acrylate could be metabolized by whole cells. Therefore, a quantity of acrylate- C^{14} corresponding to about 5200 cpm was exposed to 1 g of whole cells for 16 hr, and the extracellular acids were isolated and counted. Most of the activity recovered was in propionate and succinate (Table 42). Since lactic acid migrates with succinate, the succinate fraction was treated with KMnO_4 to destroy any lactic acid present. There was no loss of radioactivity; hence apparently no labeled lactic acid is formed from acrylate.

TABLE 42

The metabolism of acrylate by whole cells

Compound isolated	cpm
Acrylate	0
Propionate	1590
Acetate	360
Succinate	950
Succinate after KMnO_4 treatment	950

Conditions: 5220 cpm of acrylate- C^{14}
incubated with 1 g of whole cells for
19 hr at 30°C .

So far efforts to detect the participation of acrylyl CoA in reactions other than reduction to propionyl CoA have been unsuccessful. Amination of acrylyl CoA to β -alanine and hydration to lactyl and β -hydroxypropionyl CoA have been observed in other organisms⁽⁸⁻¹⁰⁾ but not in our preparations.

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EFFECTS OF DEUTERIUM ON MALIC DEHYDROGENASE

John F. Thomson, David A. Bray,* and Jacqueline J. Bummert

In a previous report some data on the effects of deuterium on malic dehydrogenase were reported.⁽¹⁾ In these experiments the Michaelis constants and maximum velocities were measured for DPNH and DPND (reduced by hydrosulfite) in both H₂O and 99% D₂O. We have obtained additional information on the effects of deuterium in this system by the use of the α - and β -stereoisomers of DPND.

Methods

Five samples of DPNH were used in these experiments. These included: 1) DPNH, a commercial sample (Sigma); 2) α -DPND, prepared by reduction of DPN with CD₃CD₂OD (a gift of Dr. V. Mohan of the Chemistry Division) in the presence of alcohol dehydrogenase; 3) β -DPND, prepared by enzymatic reduction of DPN-4-d with CH₃CH₂OH; 4) DPND₂, prepared by enzymatic reduction of DPN-4-d with CD₃CD₂OD; 5) α,β -DPND, prepared from DPN by hydrosulfite reduction in D₂O. The deuterated DPN (DPN-4-d) was obtained by formation of the cyanide-addition compound in D₂O. In this compound, the hydrogen on the 4-carbon of the pyridine ring is labile, and readily exchanges with deuterium, so that when the cyanide compound is then decomposed, the regenerated DPN contains an atom of deuterium in this position.

The enzyme (a gift from Dr. Robert W. Swick) was a preparation from Propionibacterium shermanii. The assays were carried out spectrophotometrically at 25°C, with a Beckman spectrophotometer equipped for automatic recording of the optical density changes. The assay system consisted of 0.016 M tris buffer, pH 7.4, 0.001 M oxalacetate, 0.003 M MgCl₂, 50 to 100 μ g of enzyme, and varying concentrations of DPNH.

When D₂O was used as the solvent, the buffer was adjusted to pD 8.0 (meter reading of 7.6) since we preferred to compare activities at maximum values rather than at the same pH (or pD) values.

The concentration of oxalacetate was sufficient to saturate the system, so that the data could be analyzed by the Lineweaver-Burk transformation

$$\frac{1}{v} = \frac{1}{V_m} + \frac{K_m}{V_m} \cdot \frac{1}{C}$$

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where v is the reaction velocity measured for a given coenzyme concentration C , V_m is the maximum velocity, and K_m is the Michaelis constant, the exact meaning of which depends on the mechanism of action. All values for V_m and K_m were calculated by the method of least squares.

Since the preparation designated α, β -DPND represents an equimolar mixture of the α - and β -stereoisomers, the velocity equation can be written:

$$v = \frac{V_\alpha C_\alpha}{C_\alpha + K_\alpha (1 + C_\beta/K_\beta)} + \frac{V_\beta C_\beta}{C_\beta + K_\beta (1 + C_\alpha/K_\alpha)} \quad .$$

When $C_\alpha = C_\beta = 0.5 C_t$ (the total substrate concentration) the Lineweaver-Burk transformation becomes

$$\frac{1}{v} = \left(\frac{K_\alpha + K_\beta}{K_\alpha V_\beta + K_\beta V_\alpha} \right) + \left(\frac{2K_\alpha K_\beta}{K_\alpha V_\beta + K_\beta V_\alpha} \right) \cdot \frac{1}{C_t}$$

where the subscripts α and β refer to the individual K_m and V_m values for α - and β -DPND. Therefore, for the α, β -mixture, the apparent V_m is equal to

$$[(K_\alpha V_\beta + K_\beta V_\alpha)/(K_\alpha + K_\beta)]$$

and the apparent K_m is equal to

$$[(2K_\alpha K_\beta)/(K_\alpha + K_\beta)] \quad .$$

Thus the data obtained from the individual stereoisomers can be used to calculate the K_m and V_m values for the mixture. The isotope effect is given by

$$v_H/v_D = [(V_H C_H)/(C_H + K_H)] / [V_D C/(C_D + K_D)] \quad .$$

When the substrate concentrations are equal and large in respect to K_D or K_H , the expression simplifies to

$$v_H/v_D = V_H/V_D \quad .$$

However, when C is small in respect to K , the limiting expression is then

$$v_H/v_D = (V_H/V_D)(K_D/K_H)$$

so that a larger effect on K_H/K_D than on V_H/V_D would indicate an inverse isotope effect at low values of C .

Results and Discussion

Table 43 shows the Michaelis constants and maximum velocities for the five samples of DPNH in H_2O and in D_2O .* In H_2O , there was a maximum isotope effect (V_H/V_D) of a little less than 2 (V_H/V_D) with α -DPND, but with β -DPND there was if anything, a slight inverse effect. These results would indicate that this enzyme is α -stereospecific. The data obtained with the doubly deuteriated sample, $DPND_2$, did not differ appreciably from those for α -DPND, an indication that β -substitution of deuterium had little effect on the reaction. For all four deuteriated samples, there was less effect on the Michaelis constant than on the maximum velocity.

TABLE 43
Kinetic and solvent effects of deuterium on malic dehydrogenase

Coenzyme	Isotope effects in H_2O					Isotope effects in D_2O					Solvent effects	
	No.	Km	Vm	K_H/K_D	V_H/V_D	No.	Km	Vm	K_H/K_D	V_H/V_D	K_{H_2O}/K_{D_2O}	V_{H_2O}/V_{D_2O}
DPNH	62	1.730 ± 0.049	28.7 ± 0.44	-	-	32	2.230 ± 0.110	18.9 ± 0.52	-	-	0.74	1.51
α -DPND	35	1.016 ± 0.049	15.5 ± 0.23	1.70	1.85	14	1.170 ± 0.138	10.2 ± 0.38	1.90	1.52	0.86	1.52
β -DPND	34	2.108 ± 0.089	29.6 ± 0.73	0.82	0.97	14	1.480 ± 0.166	17.3 ± 0.77	1.51	1.09	1.42	1.71
α, β -DPND Observed	21	1.366 ± 0.033	20.4 ± 0.29	1.27	1.41	25	1.435 ± 0.054	13.0 ± 0.20	1.55	1.45	0.95	1.57
Predicted	-	1.371 ± 0.048	20.1 ± 0.58	1.26	1.43	-	1.307 ± 0.108	13.3 ± 0.52	1.71	1.42	1.05	1.51
$DPND_2$	27	1.144 ± 0.058	14.6 ± 0.26	1.51	1.97	22	0.627 ± 0.113	9.0 ± 0.29	3.56	2.10	1.82	1.62

Values for Km are in moles ($\times 10^{-5}$) with standard errors; those for Vm are micromoles/min/mg N. "No." refers to the total number of experimental points, not the number of experiments.

However, in D_2O the situation was considerably different, in that the effects on Km were greater than those on Vm. This observation means that at low coenzyme concentrations, there would be inverse isotope effects in D_2O .

It is also of interest to note that the influence of β -substitution of deuterium is more important in D_2O than in H_2O , since there were significant differences between the Km values for DPNH and β -DPND on the one hand, and between those for α -DPND and $DPND_2$ on the other; i.e. there is a definite secondary isotope effect in D_2O that is not seen in H_2O .

The solvent effects of D_2O on maximum velocity were nearly the same for all substrates. The effects on the Michaelis constants were pronounced for β -DPND and $DPND_2$, but rather slight (and in the opposite

*The numbers originally reported(1) for DPNH and α, β -DPND are wrong because of an erroneous blank correction.

direction) for α -DPND, α,β -DPND, and DPNH. It should be remarked that this procedure of measuring solvent effects at the pH or pD of maximum activity has been criticized by Srere et al.,⁽²⁾ who prefer to make comparisons at the same pH or pD. Their reason is that pD "has a firm and simple significance as the activity of the cationic form of the hydrogen isotope..." However, pOD should have similar significance; and when pH equals pD, pOH does not equal pOD, since the dissociation constants of D₂O and H₂O are considerably different.

These experiments do not give a complete picture of the effect of deuterium on reactions requiring DPNH since only one concentration of substrate (oxalacetate) was used. Furthermore, since the mechanism of action of this enzyme is not known, it is impossible to state whether K_m represents the dissociation complex for the reaction:

(where E is concentration of enzyme, S concentration of substrate and C concentration of cofactor) or whether it is a function of several kinetic constants. If the reaction obeys an equilibrium mechanism rather than a steady-state mechanism, then the effect of deuterium ($K_{DPNH}/K_{\alpha\text{-DPND}}$) is very similar to that reported by Shiner et al.⁽³⁾ for yeast alcohol dehydrogenase.

A more detailed study of the kinetic and solvent effects of deuterium on lactic dehydrogenase is now in progress.

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EFFECTS OF BUBBLING ON CELL SUSPENSIONS

Robert B. Webb

In experiments involving irradiation of bacteria with the gaseous environment controlled by bubbling the gas through a suspension of cells, a substantial drop in viable count was observed in the unirradiated controls. Since this technique of gas exchange is used in several laboratories, factors involved in this bubbling loss and its effect on X-ray sensitivity were examined.

Three bacterial types were used: Escherichia coli B/r, Staphylococcus aureus FDA 209, and spores of Bacillus megaterium (ATCC 8245). The spore preparation is that developed and used by Powers et al.⁽¹⁾ E. coli and S. aureus were grown and plated on nutrient agar (Difco) at 37°C and 30°C, respectively. Spores were plated on MRVP (Difco) medium containing 1.5% agar and incubated at 37°C. Triple-layer plates were used throughout. Macroscopic colonies were counted after their appearance.

The gases used were air; oxygen, 99% dry (Matheson); nitrogen, H. P. (Matheson); helium, Grade A (National Bureau of Mines). The gases were bubbled through a column of water before passing through the cell suspensions. The suspensions contained 10^3 to 10^6 cells/ml.

The gas exchange and irradiation chambers were 30-ml buchner funnels covered with thin aluminum foil. Aliquots were removed during the bubbling or X-ray experiments, appropriately diluted, and plated in triplicate.

The X-ray source was a Maxitron X-ray tube operated at 250 kvp with 1 mm Al added filtration. The dose rate obtained was about 4000 r/min. The X-ray tube was positioned above the gassing chamber for the irradiations.

The general effect of bubbling nitrogen through an aqueous suspension of cells is shown in Figure 20. Although the shape of the bubbling effect curve differs, in each case the colony count drops below 50% in less than 15 min of bubbling. The effect of rate of bubbling and bubble size (pore size of frit) is shown for E. coli B/r in Figure 21. The rate of bubbling loss is directly proportional to flow rate of the gas for a given bubble size and is inversely proportional to bubble size for a given flow-rate. Thus the rate of cell loss seems to be related to the area of the gas-liquid interface in the suspension.

Figure 20

The relationship between viable cell count and bubbling time in aqueous suspensions of *E. coli* B/r, *S. aureus* and spores of *B. megaterium*. Nitrogen was bubbled through an "M" frit at 150 ml/min.

Figure 21

The relationship between viable cell count and bubbling time in aqueous suspensions of *E. coli* B/r under different conditions of bubbling rate and bubble size of nitrogen.

Helium and air also bring about a drop in cell count (Figure 22); however, the rate of loss appears to be less for air than for the other gases. Oxygen is less efficient than air in producing a drop in viable count. Counts of the total number of cells with a Petroff-Hausser chamber indicate that the total cell number also drops, but perhaps less than the viable count. The drop in viable count was substantial even at very low rates (e.g. 1.5 cm³/min). Gelatin was found to reduce substantially the rate of bubbling loss.

Figure 22

The relationship between viable or total cell count and bubbling time in aqueous suspensions of *S. aureus* using different gases.

This reduction in cell count is due, in part, to the cells adhering to the fritted glass since many of the cells can be placed back in suspension by drawing some of the liquid into the glass frit and expelling it. This adherence of the cells to the frit is not due to passive settling onto the frit since a drop in viable count was not detectable over the times involved if the flow rate was reduced to zero. The mechanism for the process is unknown but may involve an induced surface charge or stickiness on cells that are exposed to a gas-liquid interface. The cells would then tend to stick to the frit and walls of the chamber. However, bubbling may simply increase the probability of encounter with the glass frit. In addition some inactivation of the cells may occur (Figure 22). This effect may be related to similar reports for proteins and viruses.⁽²⁾

A similar drop in viable count associated with bubbling has been observed independently by Tallentire for spores of *Bacillus subtilis*.⁽³⁾

The effect of this bubbling loss on X-ray survival curves of *E. coli* B/r is shown in Figure 23. Although with these conditions the bubbling effect is not large, the apparent oxygen enhancement ratio of 2.7 becomes 3.4 when corrected for bubbling loss. This difference would be much larger if the dose rate were lower or if the cells were more sensitive to bubbling loss.

Other results indicate that bubbling does not alter the X-ray sensitivity of either *S. aureus* or bacterial spores. A suspension which has been reduced to 5% of its initial viable count by preirradiation bubbling still shows the same X-ray sensitivity as a non-bubbled suspension.

Figure 23

X-ray dose-survival curves of *E. coli* B/r (WP 2) in oxygen-bubbled or nitrogen-bubbled aqueous suspensions. The "corrected" values were calculated by using bubbled unirradiated suspensions as reference controls.

From these results it is evident that dose-survival curves obtained from cell suspensions with the gaseous environment controlled by bubbling may contain substantial errors. This error can be largely corrected if bubbled unirradiated controls are run in parallel with the experimental material.

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GLYCEROL AND WATER EFFECTS ON X-RAY
SENSITIVITY IN STAPHYLOCOCCUS AUREUS:
EVIDENCE FOR SURFACE ADSORPTION

Robert B. Webb

Although glycerol protection against X-ray damage in biological systems has been the subject of several recent investigations, the chemical or physical basis of this protection has not been established. Marcovich⁽¹⁾ found the protection of various alcohols to be proportional to the molar concentration. He also observed that glycerol produced about the same protection as anoxia in Escherichia coli and suggested that glycerol protects sensitive sites from oxygen sensitization to X-rays. Rosenberg⁽²⁾ showed that in yeast, glycerol shows maximum protection at high concentrations and protection roughly parallels the removal of water from the cells. Dewey⁽³⁾ demonstrated that the protective effects of anoxia and glycerol against X-ray damage in Serratia marcescens are, to a large extent, additive, suggesting different mechanisms for the two effects. Glycerol and other alcohols have also been found to protect protein molecules in solution against X-ray effects.^(4,5)

This report presents data suggesting a surface adsorption mechanism of glycerol protection and a major role for energy transfer through "bound" water in X-ray damage mechanisms. These findings further indicate that some of the mechanisms that have been identified in the bacterial spore⁽⁶⁾ may be operative in this vegetative system.

Materials and Methods

Staphylococcus aureus FDA 209 was used in the investigation. Experimental strains were maintained on nutrient agar (Difco) at 30°C. Suspensions prepared from 2- to 4-day agar slant cultures were mechanically shaken and examined microscopically for clumps. Those showing less than 90% single cells were discarded. Cells were suspended and diluted in 0.02 M potassium phosphate buffer containing 0.13 M NaCl at pH 7 for the irradiations. After treatment the surviving fraction was determined by plating (triple layer) in nutrient agar, and visible colonies were counted after incubating for 48 hr at 30°C. Direct counts of the cells in the initial suspension with a Petroff-Hausser chamber indicated that 80% to 90% of the nonirradiated resting cells were capable of producing visible colonies. There was no loss of plating efficiency in glycerol concentrations up to 85%. Viability was somewhat reduced and variable at 95% glycerol.

Irradiations were performed with a Maxitron X-ray tube operated at 250 kvp with 0.30 mm Al added filtration (HVL ~ 1.5 mm Al).

Samples were irradiated in vacuum-tight stainless steel containers 5 cm in diameter and 9.5 cm high with a window of 0.002 mm Al held in place by a detachable "O" ring seal. The chamber has a side arm which can be attached to a manifold for gas exchange. For irradiations 0.3-ml aliquots from a dilution series were placed in plastic cups 1.5 cm in diameter, and these were placed in groups of five at the bottom of the chamber. For the anoxic experiments, the chamber was evacuated to 5 mm Hg and refilled with "high purity" nitrogen which had been bubbled through water and passed over copper turnings at about 600°C. This evacuation process was repeated 8 times over a 40-min period. Loss of water by evaporation, although not extreme, was measured and taken into account. After irradiation the contents of the plastic cups were individually dumped into petri dishes without additional dilution, and pour plates were prepared. This procedure minimizes dilution errors and makes possible reproducible survival curves.

Lyophilized cells were prepared as follows. Two-tenths-milliliter aliquots from a dilution series were pipetted into 1.5-cm plastic cups to which was added 0.1 ml of concentrated nutrient broth and glucose. The final concentration was 3.2% dehydrated nutrient broth (Difco) and 2% glucose. The suspensions were quick-frozen in liquid nitrogen and dried under reduced pressure (lyophilized) for about 20 hr. The final pressure was about 10^{-4} mm Hg. The water content of the dried material was not determined. Control recovery in these experiments was about 75% of the undried controls.

When glycerol was used it was added to the cell suspensions at least 60 min before the X-ray exposures. However, the cells appeared to reach equilibrium in less than 15 min after suspension in glycerol since standing for several additional hours failed to increase their radiation resistance further. This observation is in accord with previous radiation results in other systems.^(1,2) Mitchell and Moyle⁽⁷⁾ reported that the cell membranes of S. aureus and E. coli are freely permeable to glycerol, equilibrium being achieved in a few minutes. After irradiation, suspensions in high glycerol concentrations were diluted in stages to avoid damage to the cells.

The following expression is used to describe the dose-response relationship:

$$S = e^{-kD}$$

In this expression S is the fraction surviving, D is the radiation dose and k is the slope of the dose response curve. This slope (k) is reciprocal

kilorontgens (kr^{-1}) is used as the measure of radiation sensitivity. The slopes and their standard errors were calculated by the method of least-squares. This curve-fitting technique is the same as that used by Powers et al.⁽⁶⁾ for spores of Bacillus megaterium except that a y-axis intercept of unity is assumed in these calculations. The use of this expression and the inactivation constant k is the same in concept as that expressed in the spore studies.⁽⁶⁾

Results

The effect of glycerol concentration on the X-ray sensitivity of S. aureus is shown in Figure 24. Maximum protection is approached only at very high concentrations of glycerol. The ratio of the sensitivity in buffer ($k = 0.22 \text{ kr}^{-1}$) to the sensitivity in 85% glycerol ($k = 0.024 \text{ kr}^{-1}$) is 9.2 to 1, an effect considerably larger than that previously reported for other systems;^(1,2,3,8) however, in the studies of Marcovich,⁽¹⁾ Dewey,⁽³⁾ and Hollaender et al.,⁽⁸⁾ glycerol concentrations above 20% were not studied.

Figure 24

The effect of glycerol concentration in water on the X-ray sensitivity (k) of Staphylococcus aureus (filled circles). In some cases the standard errors are smaller than the points. A Langmuir adsorption isotherm plot of the glycerol effect [$(k_0/kg) - 1$] against the concentration of Glycerol (open circles) is shown. The vertical bars are estimates of standard errors.

The combined effects of glycerol and anoxia on the X-ray sensitivity of S. aureus are shown in Figure 25, curves V and VI. In contrast to the findings of Marcovich⁽¹⁾ for Escherichia coli and Dewey⁽³⁾ for Serratia marcescens, glycerol protection in this system is much greater than protection afforded by anoxia. Removal of oxygen from the buffer suspension decreases the radiation sensitivity from 0.22 kr^{-1} to 0.092 kr^{-1} , a factor of 2.3, whereas the addition of 64% glycerol to the suspension in air decreases the sensitivity from 0.22 kr^{-1} to 0.026 kr^{-1} , a factor of 8.3. The oxygen sensitization effect is not completely removed in 64% glycerol, but is reduced from a ratio of 2.3:1 to 1.6:1, a result qualitatively similar to that reported by Dewey⁽³⁾ for S. marcescens. These results indicate

that the actions of oxygen and glycerol are partially independent. The maximum protection ratio observed in this system, the ratio of the sensitivity of the cells in buffer and oxygen to the sensitivity in glycerol and anoxia, is 13.

Figure 25. Some effects of glycerol, water and oxygen on the X-ray dose-survival response of Staphylococcus aureus.

The X-ray sensitivity of lyophilized S. aureus both in air and in nitrogen anoxia is shown in Figure 25, curves III and IV. The radiation sensitivity under this condition ($k = 0.039 \text{ kr}^{-1}$) is much less than for aqueous suspensions. However, the value of k is not as low as that obtained by high glycerol concentrations ($k = 0.024 \text{ kr}^{-1}$). No oxygen effect was observed in lyophilized cells under these conditions. Rehydration of the lyophilized material before irradiation resulted in a k of 0.14 kr^{-1} , which is only slightly less than that for cells in buffer (e.g. $k = 0.22 \text{ kr}^{-1}$). Preliminary results with S. aureus dried from distilled water on millipore filters following a modification of the technique described by Powers et al.⁽⁹⁾ indicated that the X-ray sensitivity was not substantially altered. The pronounced increase in resistance after lyophilization has been seen only if the cells were dried from a suspension containing a substance such as glucose or peptone.

Discussion

The observation that maximum protection is approached only at glycerol concentrations higher than 50% suggests that much of the protection may involve a direct and simple competition with water for adsorption sites inside the cell. The inactivation constant k at a particular concentration of glycerol may be represented as composed of two components.

$$k = k_g + k_{\min} \quad (1)$$

Where $k_{\min}$ represents that fraction not affected by glycerol while k_g is the fraction that depends upon glycerol concentration. The glycerol concentration effect data through 85% (Figure 24) are fitted by the Langmuir adsorption equation:(10)

$$\frac{k_0 - k_g}{k_0} = \frac{bg}{1 + bg} \quad (2)$$

where g is the percent glycerol concentration in solution, k_0 is the inactivation constant in the absence of glycerol and b is a constant of the Langmuir adsorption equation. From Equation 2 it follows that

$$(k_0/k_g) - 1 = bg$$

Figure 24 shows a plot of $(k_0/k_g) - 1$ against percent glycerol, assuming that $k_{\min}$ equals 0.012 kr^{-1} . This value of $k_{\min}$ gives the closest approximation to a straight line in this plot and should, therefore, be the best estimate for $k_{\min}$, the residual inactivation constant. An alternate method of plotting the Langmuir adsorption equation allows the sensitivity at "infinite" glycerol concentration to be calculated directly from the extrapolated y axis intercept. A value of $k_{\min}$ of 0.011 kr^{-1} was obtained by this method. The glycerol protection ratio based on this value of $k_{\min}$ becomes 20, suggesting that the part of the radiation damage unaffected by glycerol is indeed small. It is unlikely that the damage unaffected by glycerol can be equated with direct action.

First or second order kinetics do not describe these glycerol concentration results. This is in contrast to the findings of Dewey,(3) which were interpreted as second order kinetics. However, Dewey's results, which covered only the range to 2.0 molar concentration, are fitted by the Langmuir equation.

Water associated with macromolecules of the cells is an important part of their structure and involved in their function.(11) The possible importance of energy transfer through water bound to macromolecules in X-ray effects in bacterial spores has been presented by Powers et al.(6,12) Common properties of alcohols and water suggest that glycerol may replace

part of this structural water and thereby alter radioresistant properties. The hydroxy group permits the association or competition of alcohol and water molecules through the formation of hydrogen bonds. Replacement of the water lattice layer around macromolecules might account for radiation protection in two ways. One, the macromolecules of the cell may be more resistant to structural disruption or disorganization when glycerol is incorporated in the surrounding sheath. Two, sensitive molecules may be insulated from excited species (or free radicals) produced in nonessential molecules by the alteration or partial replacement of a "conductive" water lattice layer by glycerol. Although a major part of the X-ray damage associated with "the oxygen effect" is obviously prevented by the presence of glycerol, these results rule out the possibility that glycerol protection in this system is due solely to this mechanism as has been previously suggested for Escherichia coli.⁽¹⁾ First, the radiation sensitivity in buffer under nitrogen anoxia is much greater than in glycerol in air. Second, radiation sensitivity in glycerol is further reduced by the removal of oxygen. Dewey⁽³⁾ reached a similar conclusion based on his findings that in Serratia marcescens the effect of increasing concentrations of glycerol was almost the same for 100% oxygen, 1% oxygen and nitrogen anoxia.

The failure of the lyophilized material to show an oxygen effect was unexpected in view of results with the dry bacterial spore.⁽⁶⁾ The problem may be one of diffusion since the lyophilized material is about 3 mm thick; however, other instances of this kind have been reported.⁽¹³⁾ Experiments under way using cells dried on membrane filters without a protective agent should help answer this question.

These data are consistent with an association between water and the protective action of glycerol. However, the dehydration model of glycerol protection presented by Wood⁽¹⁴⁾ is untenable since it assumes that dehydration per se protects against X-ray damage. But the simple removal of water does not always make cells more resistant to X-rays. Seeds,⁽¹⁵⁾ bacterial spores,^(16,17) yeast⁽¹³⁾ and vegetative bacteria⁽¹⁸⁾ have not shown greater resistance after dehydration in the absence of a protective agent. Furthermore, yeast⁽²⁾ and vegetative bacteria⁽¹⁾ have not been protected by dehydration produced by concentrated NaCl. Contrary results have usually involved drying in the presence of some agent such as peptone,⁽¹⁹⁾ gelatin⁽²⁰⁾ or peptone and glucose in this report. Drying in the presence of such agents probably brings about increased local concentration of these materials inside the cells which may account for the increased radiation resistance observed.

Molecules and viruses show an increased resistance to ionizing radiations in the dry state. Also, certain molecules within cells are most resistant when the cells are dry at the time of irradiation.⁽²¹⁾ However, other molecules including those essential to the survival of the cell have

not shown a greater sensitivity in the wet cell. It is therefore difficult to assign a major role to products from the radiation decomposition of water in cell survival effects.

It is, therefore, proposed that the action of certain protective agents against ionizing radiations can be considered in terms of physical adsorption on molecules and surfaces within the cell. Experiments are in progress to characterize this group of protective agents further and to assess the role of water in radiation damage.

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ELECTRON MICROSCOPY OF MITOSIS
IN THE GIANT AMOEBA, PELOMYXA ILLINOISENSIS

Edward W. Daniels

A study was made of various mitotic stages in the radiosensitive multinucleated amoeba, Pelomyxa illinoisensis. Interphase nuclei are characterized by uniformity in size and shape; they are spheroids with diameters of about 12 to 14 μ and contain discoid and rectangular peripheral granules (nucleoli) on the inner surface of the nuclear membrane. All of some 200 nuclei in a given amoeba go through mitosis simultaneously. The major morphological change seen in the prophase nucleus is the gradual dissolution of the nucleoli. Some clumping of nucleoli along the nuclear membrane is occasionally seen. Many amoebae entering into mitosis have two kinds of mitochondria, normal mitochondria of the conventional type and those with complex internal patterns characterized by wavy, parallel tubules. As the nucleoli disappear in prophase, small spheroidal chromosomes become visible in the central portion of the nucleus. These become aligned in a plate within the nucleus during late prophase and early metaphase. The spindle fibers appear at this time and extend from the chromosomes toward the nuclear membrane. The nuclear membrane breaks up in prophase, and short fragments of it are later found distributed throughout the area of the chromosomes and spindle fibers. At metaphase the mitochondria line up at the ends of the spindle fibers distal to the chromosomes and remain in this position until the end of anaphase. At anaphase the chromosome plates separate, and the spindle fibers can be seen between the separating plates as well as between the chromosomes and the polar mitochondria. Later, the new nuclear membrane is formed around the chromosomes, apparently in part from fragments of the old nuclear membrane. Transformation from anaphase plate to telophase nucleus is gradual; during this process the spindle fiber connections are lost, and the telophase nucleus, elongated with a complete nuclear membrane, fills out into a spheroidal, young interphase nucleus. Peripheral nucleoli gradually become prominent in early interphase and eventually enlarge and assume the block-like, rectangular, and spheroidal shapes characteristic of nucleoli in mature interphase nuclei. The duration of the major part of the mitotic process in these amoebae is about one hour. The major change in nuclear shape from the telophase form to the young spheroidal form occurs after cell division.

ELECTRON MICROSCOPIC OBSERVATIONS OF THE EFFECT OF X-IRRADIATION ON THE CEREBRAL CORTEX OF THE RAT

Thomas F. McDonald* and F. Wassermann

Light microscopic investigations of radiation-induced injury in the central nervous system have led to a controversy as to the primary cause of damage.^(1,2) Some authors believe that the neuronal damage is secondary to the injury of capillaries and neuroglia, while others feel the neurons react directly to the radiation and are the primary cause of the neural damage that follows. Electron microscopic investigations into this problem are lacking.

It is our purpose to describe the changes, as seen in the electron microscope, that take place in the cerebral cortex of the rat following a single dose of X-irradiation.

Materials and Methods

Ten male Sprague-Dawley rats weighing 90 to 100 g were subjected to 26,000 r of X-irradiation localized to the surface of the skull by means of a 1-cm cone at a target distance of 28 cm.

The radiation factors were 250 kvp, 30 ma, 3.12 mm Al filtration, HVL 0.62 mm Cu. The penetration-in-depth dose through 1.5 cm of tissue was approximately 80%, and the average dose rate was about 610 r/min.

Tissues were taken from the cerebral cortex of animals up to the 8th day following irradiation. They were fixed in 2% osmic acid, dehydrated in ethanol, and embedded in Epon. Control tissue from rats of the same sex and weight was prepared in the same manner as the tissue from the experimental animals. The sections were cut with a Porter-Blum microtome and observed with an RCA-EMU-3 electron microscope.

Observations

In many areas of the cortex of animals taken 8 days following irradiation, the tissue is almost completely destroyed, as in Figure 26. Except for neurons and capillaries, there is little evidence of the numerous tightly packed cell types that occupied this area only 1 week before. The central nervous tissue, which is well known to have a minimum of extracellular space, now has large separations among the few native cells that remain. Cellular fragments and extravasated erythrocytes scattered throughout these regions indicate the severity of the damage that has occurred.

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Figure 26. From cerebral cortex 8 days after irradiation. Two large phagocytes showing cytoplasmic projections (arrows) and osmiophilic inclusions are seen in the region of a capillary (C). The surrounding tissue space is cluttered with broken membranes (M), an erythrocyte (e), and other cellular fragments.

The most numerous cells in the area at this time are large phagocytes. They have finger-like cytoplasmic projections at their periphery, and contain dense osmiophilic inclusions surrounded by cytoplasmic membranes. Their projections are often seen in close contact with the cellular fragments in the tissue spaces, as if enclosing or "grasping" them. These cells are very likely the gitter cells already described with the light microscope as occurring in lesions of the central nervous system.

In Figure 27, two smaller phagocytes can be seen in the area of a capillary. They contain inclusions similar to those found in the phagocytes in Figure 26, and the lower borders of both of these cells show cytoplasmic projections. It is interesting that the central phagocyte is located within a basement membrane, a region normally occupied only by the perivascular cell. It is also significant that the basement membrane is absent from the same cell-type in the tissue space to the right. Such observations suggest a developmental relationship between perivascular cells and phagocytes.

Figure 27. From cerebral cortex 8 days after irradiation. The plasma membrane of the neuron (N) is absent, and the cytoplasmic contents seem to be moving into the tissue space. The central phagocyte is surrounded by a basement membrane (B), and shows cytoplasmic projections (arrow) at its lower border. Another phagocyte is seen in the tissue space at the right.

Many of the neurons at this time appear to maintain their normal structure, but others are found in various states of disintegration. For example, the plasma membrane at the surface of the neuron in Figure 27 has apparently ruptured, and the cytoplasmic contents seem to be spread out into the tissue space. The endothelial cells of the capillaries in these areas occasionally contain osmiophilic inclusions, but other than this their structure is similar to that found in controls.

The events leading to the damage seen in Figures 26 and 27 can be found in cortical tissue taken during earlier periods following irradiation. The earliest indications of damage are seen in the capillaries 24 hr after irradiation. Although relatively few of them are involved, those that are show injuries ranging from thrombosis to complete disruption. In the cortex of animals taken on the 2nd and 3rd days, one sees, in addition to the capillary damage, swollen astrocytes that may contain fragments of erythrocytes or abnormal accumulations of granules as in Figure 28. Although the neurons often show irregular indentations in their nuclei at this stage, their basic submicroscopic structure is similar to that of the control neurons.

The perivascular cells of some of the capillaries begin to show changes during these first 3 days. They enlarge within their basement membrane, and their cytoplasm often contains osmiophilic inclusions, as well as peripheral projections, in a manner similar to the previously described phagocytes.

Sections taken on the 4th day after irradiation bridge the gap between the two stages thus far presented. In the more severely damaged areas of this tissue, as shown in Figure 29, there is an increase of cell forms. This is the result of three factors; many of the astrocytes now show moderate to extreme swelling, extravasated erythrocytes are common, and phagocytes containing numerous inclusions appear. Further, in regions not far from that shown in Figure 29 the extracellular space is enlarged. During the earlier periods this space remains a rather normal 150- to 200-Å gap, but at this time areas up to 2μ can be found to separate adjacent cells.

Many of the neurons continue to show indentated nuclei, and a few of them may show swelling and inclusions, but on the whole the neurons as well as the capillaries found in this tissue are apparently normal. The phagocytes are dispersed throughout the tissue and are often seen in contact with capillaries. Their inclusions vary greatly in form; many of them seem to be myelin figures undergoing dissolution.

In all of the sections of irradiated material taken for this study it was obvious that certain areas were more damaged than others. The damaged areas were microscopic in the early stages, measuring about 50μ in diameter, but they became progressively larger in the later stages.

Figure 28. Cerebral cortex 3 days after irradiation. The swollen astrocyte (A) at the top of the micrograph shows an abnormal accumulation of granules, and to the right it borders on a damaged capillary (C). Except for an indented nucleus, the neuron (N) is not essentially different from those found in control tissue. Several axons marked by surrounding myelin (arrows) are found among less swollen astrocyte processes (ap).

Figure 21. Cerebral cortex 4 days after irradiation. Extremely swollen astrocytes are seen at the top of the micrograph. Phagocytes (arrows) are recognized by their osmiophilic inclusions. Extravasated erythrocytes (e) appear to be within the cells surrounding the capillary (C). Numerous astrocyte processes are noticed in various stages of swelling. The two cells at the bottom may represent damaged neurons or oligocytes.

Discussion

This study shows that after receiving 26,000 r of X-irradiation the capillaries, astrocytes and neurons of the rat cerebral cortex become damaged in a sequential manner.

During the first few days the capillaries exhibit the most immediate and severe damage. Although relatively few capillaries were affected, it must be kept in mind that the electron microscope presents a very small field, and it is difficult to estimate the over-all damage for this period. This early breakdown in capillaries may be the submicroscopic mechanism involved in the observation by Van Dyke et al.,⁽³⁾ that 24 hr after administering 26,000 rads to the rat brain, intravenously injected fluorescein stained the irradiated area.

Almost as early as the capillary damage was noted, astrocyte swelling could be seen, which then progressed to the exaggerated state shown in Figure 29. The gross effect resulting from this was described by Ross et al.,⁽⁴⁾ who reported that in monkeys taken early after administration of 6,000 r to the head, the edematous cerebrum bulged through incisions made in the dura. They also stated that the swelling subsided in animals taken on the 2nd week. It was not surprising that the swelling found in the cortex following irradiation occurred in the astrocytes. Torack et al.⁽⁵⁾ found, both in edematous brain tissue of mice after ingestion of alkyl tin compounds, and in that of areas surrounding human intracranial neoplasms, that the edema was due to swelling of astrocytes and not to an increase in the extracellular space.

The relative absence of astrocytes during the later periods, along with the appearance of broken membranes, organelles and nuclei in the tissue spaces, gives ample evidence that the swollen astrocytes rupture. The enlargement of the extracellular space that we see on the 4th day may be the result of cells and their processes moving into areas once occupied by astrocytes. Further, the subsiding of edema found by Ross et al.⁽⁴⁾ during the 2nd week may be explained by the release of fluids from these broken cells.

Disrupted capillaries were no longer encountered after the first 3 days, but continuing observations of extravasated erythrocytes indicated a continued impediment in the vascular system. The neurons showed little distress from the radiation until the 4th day, and at this time only a few cells were involved. As late as 8 days following irradiation many of the neurons were found to maintain their basic structural integrity.

Considering all of the circumstances that occur in this study relating perivascular cells to phagocytes, we believe that, at least under these conditions, the perivascular cells can differentiate into phagocytes.

That certain areas in the cortex are more affected by the radiation than the others may well reflect an uneven distribution of ionization within the tissue. All of the cell types must be affected by this ionization, and yet, our observations show that there is an order of expression of damage: first in the capillaries, next the astrocytes, and finally the neurons. The astrocytes, although second to show damage, are the most severely involved in the later stages. The neurons exhibit relatively high resistance to the irradiation.

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LOW TEMPERATURE STORAGE OF EPOXY RESINS USED FOR EMBEDDING IN ELECTRON MICROSCOPY

O. T. Minick

Epoxy adhesive, catalyzed and bubble-free, prepackaged and frozen by the manufacturer, has solved some real problems in the bonding of highly precise equipment produced by industry. The availability of packaged prepared material eliminates improper mixing of resin and curing agent, material waste and air entrapment, all of which adversely affect the reliability and the reproducibility of procedures for final application of the adhesive.⁽¹⁾

In electron microscopy similar problems have been encountered in the preparation of epoxy resins for embedding. Most failures with the embedding method arise from failure to mix adequately these viscous materials.⁽²⁾ By mixing the Epon mixture in large quantities, then prepackaging and freezing it until we are ready to use it, we have eliminated most of these difficulties.

Figure 30

Stirring rod used for mixing the epoxy resin.

Method

The materials were as used by Luft.⁽²⁾ The stirrer was a Cenco variable-speed Type NSI-12 obtained from Central Scientific Company. Stirring rods, shown in Figure 30, were of glass. At the working end were two propeller blades, each one and one-half inches tip to tip. One propeller was placed at the tip of the rod and the other one-half inch above it. The blades oppose each other in direction by 90°.

The embedding mixture was prepared in one-liter or one-half-liter amounts depending on demand. The procedure for 500 ml is as follows: Place 220 ml of Epon 812 in a 1-liter beaker and begin mixing; the stirrer should be set at low speed throughout the procedure. Add 185 ml dodecenyl succinic anhydride and 94 ml Nadic Methyl Anhydride* and mix for 15 min. Then add 10 ml of 2,4,6-tridimethylaminomethyl phenol and stir for 30 min. Pour the mixture into 2-ounce bottles. Line the caps with aluminum foil, tighten them securely, place in a leak-proof paper bag and store at -50°C. The leak-proof bag protects the bottles against accidental breakage and will help contain the plastic in case of spillage.

*Allied Chemical Co.

Results

Samples were removed from the freezer and tested every week for a period of 4 weeks. Tests were made for viscosity, hardness after curing, and sectioning qualities. The viscosity was unchanged even after a 4-week period. The blocks prepared from this material are a bit harder than those prepared from freshly mixed resin but no differences are apparent in the final sectioning. Further improvements in this technique are being investigated.

I wish to thank George Svihla for suggesting the low temperature storage of prepared epoxy resins for electron microscopy.

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THE FINE STRUCTURE OF MEIOTIC CHROMOSOMES IN THE PIGEON

Bernard R. Nebel and Elizabeth M. Coulon

Fawcett showed in 1956 that the pigeon is unusually favorable material for the study of spermatogenesis.⁽¹⁾ In two recent publications it was shown by us that meiotic spermatocyte chromosomes have lampbrush structure in pachytene, one of the stages preceding reduction division.^(2,3) This is in essential agreement with the observations of Moses⁽⁴⁾ on other material.

Lampbrush structure may be described as follows: When two homologous chromosomes are seen in the electron microscope at pachytene the most obvious parts are two dark parallel rods. On closer observation one sees that the parallel rods are peripherally surrounded by a mass of fine threads extending up to 500 A on all sides and apparently radiating away from the two rods. These fine threads have given rise to the term lampbrush structure since the entire structure looks like an implement used some time ago to clean the cylinders of kerosene lamps.

Certain technical terms have been introduced in the literature mostly by Moses.⁽⁴⁾ The "synaptonemal complex" refers to the entire compound structure of two paired pachytene chromosomes, and thus contains the two dark parallel rods. These are technically called the "lateral components." Each lateral component contains two parallel chromatids which may be called "sister chromatids." The fine threads radiating out from the lateral components are termed "lateral loops." Finally, we have the "midaxial space," which refers to the region between the lateral components and describes the pairing surfaces proper of the two homologous chromosomes. This information is represented in Figure 31, which is our schematic view of a piece of a bivalent pigeon spermatocyte. The diagram gives no idea of the overwhelming number of lateral loops. Only two have been drawn in full; the short stub on the lower right of the diagram suggests a lateral loop truncated by the microtome knife. Between the two lateral components is the midaxial space which shows indications of subdivision.

Three enzymes were used to ascertain the biochemical nature of the synaptonemal complexes. Trypsin and ribonuclease when used alone did not markedly affect the complexes. Deoxyribonuclease destroyed the lateral loops. When all three enzymes were used on the same material all parts of the synaptonemal complexes appeared badly corroded and were gradually obliterated. The synaptonemal complexes were also subjected to 1,2, and 3 kr of γ -irradiation at 1 and 2 days before sacrifice of the animals. No immediate effects were seen in the electron microscope.

Figure 31

Schematic drawing of a portion of two homologous chromosomes of a pigeon spermatocyte seen by electron microscopy. SC sister chromatids; LC lateral components; LL lateral loops.

Although many details will require further work, a new picture of the structure of the chromosome is beginning to emerge. First, it became clear that the lateral loops of meiotic prophase chromosomes are carried into metaphase. This finding suggested a reevaluation of chromosome structure in general. A study of onion chromosomes in somatic division was aided by the improved resolution obtainable with Epon as an embedding medium. It appears that the somatic onion chromosome contains a very great mass of very fine lateral loops present at all stages of the cycle. Thus the concept of the hierarchy of numerous semi-independent gene threads⁽⁵⁾ is in error, and the lateral loops may, in part, have been mistaken for microfibrillar elements of many parallel gene strings. It is likely that the problem of strand number in the chromosome will revert to a condition described by one of us in 1947.⁽⁶⁾ That is, the chromosome will consist of chromatids and occasionally half-chromatids, but it will be the task of the electron microscopist to define the position, orientation, and multiplication of the innumerable lateral loops.

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THE STRUCTURE OF SPINDLE FIBRILS IN A GIANT AMOEBA

L. Evans Roth and Edward W. Daniels

Dividing amoebae (Pelomyxa carolinensis) were fixed in osmium solutions buffered to pH 8.0, embedded in methacrylate, and sectioned at 20 to 100 $m\mu$ thicknesses for observation in the electron microscope. If divalent cations (0.002 M calcium, magnesium, or strontium as chlorides) were added to the fixation solution, fibrils 14 $m\mu$ in diameter and having a dense cortex were observed in the spindle. If the divalent ions were omitted, oriented particles of smaller size were present, and tubules were not obvious.

The stages of mitosis were observed and the spindle fibrils compared. Fibrils fixed in the presence of calcium ions are not as well defined in early metaphase as later, but otherwise have the same diameter in late metaphase, anaphase, and early telophase. Fibrils are surrounded by clouds of fine material except in early telophase when they are formed into tight bundles lying in the cytoplasm unattached to nuclei. Metaphase and anaphase fibrils fixed without divalent ions show no observable differences.

These observations are consistent with the hypothesis that spindle fibrils are composed of polymerized, oriented protein molecules that are in equilibrium with and bathed in unoriented molecules of the same protein. Fixation in the presence of divalent cations has probably caused an interaction of these cations with the free carboxyl groups of the spindle protein, resulting in a hyper-bound condition as compared to the in vivo protein configuration.

A METHOD FOR OBTAINING FIXING SOLUTIONS OSMOTICALLY EQUIVALENT TO PHYSIOLOGICAL SALINE SOLUTION

Theodore N. Tahmisian

With the advent of electron microscopy it became evident that tissue-fixing solutions must simulate physiological conditions with respect to tonicity. To obtain ultrathin sections without cellular distortion and intracellular distortion of organelles requires that the fixing solution must not only have optimal pH and isotonicity, but must also contain the cations found in physiological ("normal") saline solution as well as the fixing agent (e.g., osmium, chromium, manganese, lead, gold, uranium, or aldehydes). As the pH of the fixative approximates that physiologically optimal for most cells, i.e. pH 6.8, a better fixed tissue is obtained. However, the acidity or alkalinity of fixing solution has been deliberately varied from 3.5 to 9 for specific effects.

An 0.85% aqueous NaCl solution is considered "normal" for most mammalian cells and 0.9% for some insect cells. Some intracellular components require solutions of higher osmotic strength; some crystalloids require an equivalent of 1.4% NaCl. There are occasions when hypotonic and hypertonic fixing solutions can be utilized intentionally to distort cellular components, and the appropriate osmotic pressures must be obtained easily and quickly.

Once one has established the concentration of saline solution that produces the desired effect, regardless of its tonicity, it is possible to obtain a fixing solution that is equivalent in ionic strength. This can be done easily by calculation, using the dissociation constants of the components, but such a procedure does not take into account the effects of complex formation on tonicity.

A 0.9% aqueous ("normal") NaCl solution has a molarity of 0.154. Any fixing solution, regardless of its ionic composition, that will depress the freezing point to the same extent as 0.154 M NaCl will have the same number of particles in solution and by definition the same osmotic pressure. We obtain NSE (normal-saline-equivalent) fixing solutions equivalent to 0.9% saline using the freezing point depression method. The tonicity of the NSE fixative is determined using an Aminco-Bowman Freezing Point Depression Apparatus.

Standard solutions of NaCl from 0.045% (0.077 M) to 1.80% (0.308 M) were prepared, and the freezing point depression was determined for each (Figure 32). From this curve the osmotic pressure equivalents of any fixing solution can be determined and corrected to the NSE if necessary.

Figure 32

Standard curve for NaCl solution: concentration vs. lowering of freezing point.

The Palade osmium fixing solution is 1.15% NSE. The composition of the NSE solution developed by us is as follows:

0.14 <u>M</u> Na Veronal acetate solution	2	cc.
0.10 <u>N</u> HCl	2	cc.
2% OsO ₄ solution	5	cc.
9.0% NaCl solution	0.42	cc.
Water	0.58	cc.

This solution has a freezing point depression equivalent to that of 0.9% NaCl solution.

ELECTRON MICROSCOPIC EXAMINATION OF THE WALL OF THE LACUNAE AND CANALICULI IN BONE

F. Wassermann

The systems of lacunae and canaliculi in bone, which extend from the haversian canals into the mineralized bone tissue (matrix), represent the pathways for the exchange of substances between blood and bone (Figure 33). By the same routes the bone cells (osteocytes) are supported. The understanding of the mechanisms involved in the transport of materials from and to the cells and from and to the matrix depends to a great part on our knowledge of the structures of the content and the walls of lacunae and canaliculi. An electron microscope study of these structures is, therefore, at present a desideratum.

Material and Methods

Small pieces were chipped from the compact bone of femurs of adult rats or guinea pigs and from puppies of about 3 months of age and were fixed in buffered osmic acid on ice for 2 hr. The specimens, either undecalcified or after decalcification with Sodium Versenate, were embedded in methylacrylate or more recently in Epon.⁽¹⁾ The sections of both undecalcified and decalcified specimens were cut on the Porter-Blum microtome with glass knives. The microscope used was the RCA - EMU 3.

Figure 33

Part of a ground section of a human femur showing the system of lacunae and canaliculi: hc, haversian canal, hs, haversian system. kl, cementing line at the border of the haversian system; l, lacunae; cl, canaliculi, in the matrix, the concentric lamellae. From Weidenreich.⁽²⁾

Observations

For a necessary preliminary orientation in the topography of the region described in this paper two photomicrographs are presented in

Figures 34 and 35. The bone from which the section shown in Figure 34 was taken, was fixed in formalin-alcohol, decalcified and embedded in paraffin; the particular section of Figure 34 was treated with the periodic acid-Schiff (PAS) reagents for mucopolysaccharides. An osteocyte is seen in its lacuna. The relatively wide space around the cell of about 2.7μ is certainly an artifact due to the unavoidable shrinkage of the cell within its rigid encasement during fixation and dehydration. The dark lining of the lacuna, however, is a real structure long known as the capsule of the osteocyte or the Rouget-Neumann sheath (RNS), the "Grenzschleide" in the German, or "la gaine limitante" in French literature.⁽²⁾ It stains distinctly with hematoxylin, and its width, about 1μ or less, can be measured where it is orthogonally sectioned. The section of Figure 35 is from a sternum of an adult guinea pig and was made from the fresh tissue with the freezing microtome using a knife chilled with dry ice. It was examined and photographed in Ringer solution, without staining, 5 to 10 minutes after the sample had been taken from the anesthetized animal. Shrinking, if it occurs at all, cannot be considerable under these conditions. Accordingly the space around the grey cell body is not the artificial space of Figure 34. It corresponds in its width of about 0.7μ to the width of the RNS in Figure 34. Lipp,⁽³⁾ studying such frozen sections of fresh bone, has shown that the content of the pericellular space gives a positive PAS-reaction.

Figure 34

Photomicrograph of section from decalcified bone (guinea pig femur) fixed in formalin-alcohol. Periodic acid-Schiff reaction. Osteocytes are within the lacunae. Surrounding the lacunae is the bone matrix. Since the space between the surface of the cells and the dark lining (pink in the original) of the lacunae is about 2μ wide, it is certainly produced by shrinkage of the cells during fixation and dehydration. The dark lining is the Rouget-Neumann sheath (RNS) of the osteocyte; its material is PAS-positive, and it is about 0.7μ thick.

Comparison of these two light-microscope sections confirms the generally accepted opinion of the histologists⁽⁴⁾ that the osteocyte with its

processes fills the entire lacunal and canalicular spaces but for the material of the RNS. This extracellular, mucopolysaccharide-containing material is, via the stomata of the canaliculi, continuous with the connective tissue ground substance around the blood vessels in the haversian canals (see legend of Figure 33). The medium of transport in bone is, therefore, the same as in connective tissue, viz. the ground substance, which is known to occur in various structural and functional modifications (Reference 5, p. 311 ff.). This concept was proposed by Policard and Baud in 1958.⁽⁶⁾ It is, however, interesting to point out that the intercellular material in bone, in contrast to its continuous counterpart in connective tissue, is divided into two compartments: the RNS and the calcified matrix. Any material that is to enter or to leave the matrix, therefore, passes a border between the soft and the hard intercellular compartment. A study of the fine structure of the wall of the lacunae and canaliculi yields, as will be shown here, some morphological data concerning this border region.

Figure 35

Photomicrograph of decalcified bone (guinea pig sternum) prepared from fresh bone with the freezing microtome, unstained, examined in Ringer solution with the phase microscope. There is an osteocyte in the lacuna, from which several canaliculi extend into the matrix. There is no considerable shrinkage under the conditions of preparation. The space between the cell surface and the wall of the lacuna is about 0.7μ wide and corresponds therefore to the RNS in Figure 34.

The electron microscopic aspect of the osteocyte in the lacuna (Figure 36) and of its processes in the canaliculi in longitudinal (Figure 37) or cross sections (Figures 38, 39) in decalcified sections appear at the first glance very similar to the corresponding light-microscope pictures, such as that of Figure 34. However, the pericellular space is only about 0.6μ wide and, therefore, cannot be identified with the shrinkage space of Figure 34, which has a diameter of about 2.7μ . In other electron micrographs it is even narrower. It therefore corresponds to the RNS of Figure 34, which was found to be less than 1μ wide. Baud^(7,8) (and Dudley and Spiro⁽⁹⁾) also identified the pericellular space as it

appears in the electron microscope with the RNS without, however, contrasting it to the shrinkage space in the light-microscope preparations. It follows that OsO_4 fixation causes very little or no shrinkage of the bone cells, in contrast to the fixatives used in light microscopy.

The question can be raised, however, whether the dark lines that are so conspicuous as the lining of lacunae and canaliculi in Figures 36 to 38 could not be the electron microscopic equivalent of the RNS. The measurements of these lines show them to be much too narrow to be identical with the RNS, their widths varying from 200 to 300 Å as compared with 5000 to 10,000 Å for the RNS. We come, therefore, to the conclusion that the pericellular space in the electron microscopic pictures represents the RNS and that the dark lining is a hitherto unknown structure, a border region of the matrix.

Figure 36. Electron micrograph of section of decalcified rat bone. Osteocyte in lacuna. The ostium of one canaliculus was met in the section. The nucleus of the osteocyte shows the characteristic aggregations of chromatin. The pericellular space is about 0.6 μ wide. It corresponds therefore to the pericellular space in Figure 35, that is, to the RNS. The dark line at the wall of the lacuna has a diameter of about 200 to 250 Å. It cannot, therefore, be identical with the RNS, but is a lining of the lacunae and canaliculi that was not known in light microscopy.

Figure 37. Electron micrograph of section of decalcified rat bone shows part of longitudinal section of canaliculus containing a process of the osteocyte in the lacuna below. The pericellular space is only 0.2 to 0.3 μ wide, the lining of the canaliculus has a diameter of about 300 Å. Notice the central position of the process in the lumen. In the matrix the collagenous fibrils are mostly in cross section. The spaces between the fibrils are occupied by the ground substance of the matrix in which the bone crystals (see Figure 40) are embedded.

It is present in Figure 12 of the recently published paper on "The fine structure of bone cells" by Dudley and Spiro⁽⁹⁾ but was not mentioned or discussed by the authors. The observation already made by Baud⁽⁷⁾ that the cell processes occupy mostly the central portion of the canalicular lumen indicates that they are embedded in a rather viscous material, that is, the mucopolysaccharide-containing ground substance of the RNS. The presence of this material sometimes becomes visible in the form of an opaque, flocky content of the pericellular space which was also observed by Dudley and Spiro. Figure 39 presents a good illustration of this material. The cross sections of canaliculi are very small, with diameters of the order of about 0.2 μ ; we are dealing here with the peripheral part of the canaliculi. They do not contain cell processes as those in Figure 38. But they are not empty. The grey flocky material seen in the lumina is the same as often observed between the osteocyte and the wall of the lacuna. Pictures such as that of Figure 39 confirm the opinion of Lipp⁽³⁾ and others that the processes of the osteocytes retract from the peripheral parts of the canaliculi and that the material of the RNS is then the only content of the lumina.

Figure 38. Material the same as in Figure 37. The canaliculi are approximately cross-sectioned. Width of the pericellular space is about the same as that in Figure 37, the dark lining is about 150 Å wide. In the matrix are the collagenous fibrils.

In the electron micrographs of undecalcified bone sections (Figure 40) the dark lining of lacunae and canaliculi, so clearly defined in all undecalcified sections, cannot be distinguished. Evidently, the border region of the matrix, like the rest of it, is filled with bone crystals and appears in the undecalcified section equally dark. If it becomes distinguishable in decalcified sections by its greater density this can be caused only by a greater density of the ground substance which becomes apparent after the removal of the crystals.

The crystals in the border region of the matrix, although probably of the same kind as the rest of the bone crystals, are, nevertheless, of special significance because of their position at the edge of the matrix. A picture of this region in an undecalcified and thinner section at higher magnification in Figure 41 shows not only the crystals but also the cross-banded collagenous fibrils of the matrix at places where the crystals are cut off. One recognizes that the lumen of the lacuna is not lined by fibrils but by a layer of crystals, which are not packed between fibrils but are free toward the lumen and in direct contact with the content of the

pericellular space, that is, with the RNS. They seem also less well-ordered than the crystals that are fully attached to surrounding fibrils as first described by Robinson and Watson.⁽¹⁰⁾ Directly exposed to the medium of transport of substances between blood and bone these crystals in the border region represent probably the most labile part of the bone minerals. Their special situation and supposed physiological significance justifies distinguishing them from the bulk of the bone crystals as the "coastal crystals" of the bone matrix.

Figure 39. Cross section of canaliculi in decalcified section of dog bone. The width of the canaliculi of only $0.2\ \mu$ indicates that the canaliculi are sectioned at a distance from the lacuna. They contain a flocky material but no cell processes.

Figure 40. Lacuna in section of undecalcified dog bone. In the matrix are the masses of bone crystals. Where the cross-banded collagenous fibrils are visible one notices that the bone crystals are arranged with their long axes parallel to the fibrils and that they are packed in between the fibrils. The dark lumen of the lining of the preceding figures is barely, if at all, distinguishable.

Figure 41. This figure shows the structure at the border region of the matrix in section of undecalcified dog bone. The collagenous fibrils are in part denuded of crystals. One notices that the border region, which corresponds to the dark lining in the decalcified sections, consists of crystals, the "coastal crystals" described in the text. They have no attachment to fibrils at the side of the lumen and are, therefore, not as orderly in arrangement as those packed in between the fibrils.

Summary

Electron microscopic examination of sections of both decalcified and undecalcified bone revealed the existence of two layers around the osteocytes. One appears in the electron micrographs as a pericellular space whose width is of about the same order as that of the Rouget-Neumann sheath in the light microscopic sections. After osmium fixation, shrinkage of the bone cells does not seem to produce an artificial space as do the fixatives used in light microscopy. The second layer is represented in decalcified electron microscope sections by a conspicuous dark line which has diameters of only 200 to 400 Å and cannot be identical, therefore, with the Rouget-Neumann sheath. It is a new structure, not visible in the light microscope. While it is never missing in decalcified sections it cannot be distinguished in undecalcified sections since it contains bone crystals and appears therefore as dark as the rest of the matrix. It belongs to the matrix as a special border region. This region differs from the bulk of the matrix in two respects: first, the ground substance, in which the crystals are embedded, seems to be denser than the ground substance of the main part of the matrix; second, the crystals toward the lumina of the lacunae and canaliculi are not bound to collagenous fibrils and are not as well ordered as those that are tightly packed between the fibrils. The border region of the matrix is thought to have special significance for the exchange of material between the bone matrix and the transport medium of the Rouget-Neumann sheath.

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RADIOAUTOGRAPHIC STUDY CONCERNING THE ROLE OF ASCORBIC ACID IN COLLAGEN FIBER FORMATION

F. Wassermann, Walter E. Kisielewski and Lucille Agemura

The absence of fiber formation in wounds of guinea pigs suffering from experimental scorbutus and the resumption of normal healing after readministration of the vitamin has been demonstrated by numerous investigators since Wolbach's classic studies.⁽¹⁾ Although observations have been accumulated in support of the opinion that ascorbic acid deficiency affects primarily the metabolism of the fibroblast, the mechanism by which the vitamin acts in fibrillogenesis is still unknown. Several possibilities are to be considered: the fibroblast may be unable to absorb the constituents of collagen, especially proline, the most characteristic of its amino acids, it may be unable to hydroxylate this particular precursor, or it may be prevented from synthesizing and polymerizing the collagen molecule. Since the latter step in fibrillogenesis is not necessarily an intracellular process but occurs outside the cell as well,⁽²⁾ the action of ascorbic acid may also concern the ground substance which must be in a state to allow the collagen polypeptide chains to be properly assembled.

To test the first listed of these possibilities we made use of C^{14} -labeled proline, injecting it intravenously into guinea pigs that had previously been tenotomized (Achilles tendon) while suffering from experimental scorbutus. The manifestations of clinical scorbutus in the animals kept on a scorbutogenic diet for 3 to 4 weeks were loss of weight, hemorrhages in the musculature, brittle bones, and loosened teeth. Fifty microcuries of labeled DL-proline hydrochloride (1 ml volume) were injected about one week after the operation both into the manifestly sick animals and into those of a second group that had previously received 20 mg ascorbic acid intravenously on three consecutive days. The tissue of the regenerating tendon was excised from the ether-anesthetized animal at intervals of 1 to 6 hr after the proline injection, fixed in buffered formalin (pH 7.0), and embedded in paraffin; 5- μ sections were used for autoradiography. From numerous light microscope and electron microscope observations of previous experiments concerning the stimulation of fibrillogenesis in scorbutic guinea pigs by ascorbic acid, we could be sure that the tissues of the animals of the second group contained enough ascorbic acid to initiate fiber formation. Of 24 experimental animals 13 were successfully treated and survived, while one succumbed to the scorbutus despite 2 ascorbic acid injections. Of the 8 uninjected animals 6 died, so that only 2 of the first group remained for comparison of radioautographs of scorbutic tissues with those from ascorbic-acid-containing tissues. The high mortality of the animals of the first group and the recovery of those of the second is further proof that our experimental animals were suffering from severe scorbutus. While we could expect the labeled proline to be present in the

fibroblasts of the recovering animals, the question we wanted our experiments to answer was whether or not it was also to be found in the tissue of the regenerating tendon of the still scorbutic pigs.

The main result of the present series of experiments was that the radioautographs were practically alike. Our figure (Figure 42) represents a section from a scorbutic untreated animal 6 hr after the injection of labeled proline. The active material in the form of small, discrete grains is evenly distributed throughout the tissue. It is present over the fibroblasts and also, in about the same density and distribution, above the intercellular spaces. This picture is representative of all the radioautographs we have obtained in this series of experiments.

Figure 42

Photomicrograph taken with Zeiss apochromatic 40 and ocular 12. Radioautograph of section from the tissue between the stumps of the severed Achilles tendon of a scorbutic guinea pig, one week after tenotomy. The animal was sacrificed 6 hr after a single injection of 1 ml labeled DL-proline hydrochloride in a concentration of $50 \mu\text{c}/\text{ml}$. Notice the spindle-shaped fibroblasts and the intercellular material. The radioactive grains are evenly distributed throughout the section.

We can be certain that the grains represent radioactive material since the intensity of the grains in the sections is considerably higher than that found in our control emulsions. We can, therefore, say that radioactive material has accumulated in the wound tissue regardless of the presence or absence of ascorbic acid. The material was present as early as 1 hr after injection, and the number of grains increased somewhat up to the 6th hr. That the absorption of the material was dependent in some fashion on the special character of wound tissue is indicated by the relative scarcity of grains in the areas of muscle fibers or of the stumps of the tendon.

It cannot be told from radioautographs alone whether the active material was proline, and perhaps hydroxyproline, or a metabolic degradation product of the injected amino acid. In order to decide this question radioautography and chemical determination of the proline content of the tissue will be combined in further experiments.

Another point that needs further exploration is the approximately even distribution of the active material throughout the tissue. Considering the role of the fibroblast as the site of collagen synthesis one would expect that at a certain time after the injection the greater amount of the proline would be found in the cell as its transport toward the cells comes to an end. The presence of the grains over the intercellular spaces at 6 hr after the injection would indicate that at this time a considerable part of the material was still on the way to the cells. The experiments must be extended in time to find out whether or not the expected increase of intracellular material, parallel to a decrease in the extracellular, can be demonstrated. Only after this has been decided and the nature of the material has been chemically determined can we accept the conclusion suggested so far by our radiographs: that the role of ascorbic acid is not to enable the fibroblast to absorb the building blocks of collagen but, rather, it must be related to other steps in the complex process of fibrillogenesis.

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ACTION SPECTRA AND QUANTUM REQUIREMENTS FOR
PHOTOSYNTHETIC PROCESSES

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The wavelength dependency of photosynthesis in intact tissues has been frequently determined, generally with oxygen production as the index of photosynthetic efficiency. The "driving force" of this CO₂ cycle is considered to be the light-induced reaction whereby the reduction of TPN is coupled to the synthesis of ATP. This reduction and esterification is readily measured with isolated chloroplasts. It was desirable, therefore, to determine what the quantum requirements and action spectrum were for TPN reduction and associated ATP formation in the isolated system. The yields in monochromatic light were also of interest in that the ratio of ATP to TPNH was known to approach unity only at saturating intensities of white light.

Suitable suspensions of spinach chloroplast fragments containing Mg, inorganic phosphate, ADP, TPN, and pyridine nucleotide reductase were irradiated at various loci on the biological spectrograph. Light intensities and irradiances were kept to the linear-response range. The action spectra so obtained for TPN reduction and ATP formation paralleled the absorption spectrum of the chloroplast, i.e. the peak quantum efficiencies were near 450 and 675 m μ with a secondary peak in the carotinoid region at 500 m μ . The secondary peak probably represents an efficient fluorescence transfer of energy to formation of photosynthetic reductant, since the quantum yield was approximately equal to that at 475 m μ for the TPN reduction, and only slightly less for the phosphorylation. The action spectrum for photophosphorylation supported by phenazine methosulfate (PMS) in place of TPN as a reductant followed a similar pattern. In this instance, as with TPN, there were no peaks at any wavelength above 680 m μ .

Highest quantum efficiencies occurred under red light. They were about 1.4 times those obtained for the various reactions in the blue spectral region. At 675 m μ , the quantum requirement was about 9 for TPN reduction (or 18 per molecule of oxygen evolved) and about 18 for TPN-supported phosphorylation. The required quanta per molecule of ATP formed in the presence of PMS is about 10 to 15 times greater. If a light-induced phosphorylation similar to that supported by PMS in these experiments occurs in the intact cell, one may question its physiological function; the photophosphorylation associated with TPN reduction appears to be a much more efficient process.

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The quantum efficiencies for the reactions examined were not independent of wavelength in the visible spectrum between 375 and 680 m μ ; they dropped in the green region. This may reflect the lower efficiency of energy transfer by co-pigments in the isolated particles. The ratios of ATP formed to TPN reduced at various wavelengths were then determined for 2 light fluxes differing 8-fold in intensity. These ratios were below unity. Ratios at the lower intensity were roughly half of those at the higher. The latter only approached 1 in the blue spectral region. Either the coupling of phosphorylation to nucleotide reduction is non-compulsory, or there is an interaction of different photochemical processes at different wavelengths. The bases for the drop of quantum efficiency in the green, and the light-intensity dependence of the ATP/TPN ratio, will be investigated in subsequent studies.

INACTIVITY OF HEXITOLS AND HEXOSES IN THE BIOSYNTHESIS OF AUXIN FROM TRYPTOPHAN

Solon A. Gordon and Evelyn Buess

The composition of coconut milk is of considerable interest since this liquid endosperm supplies the requirements for the growth of many tissues and organs in vitro. Pollard et al.⁽¹⁾ have classified the constituents of the milk largely on the basis of their function either as growth substrates or as growth regulators. In the first group may be placed the sources of carbon and nitrogen, and the inorganic nutrients. Compounds of the regulatory group were separated into two classes: a) "Neutral component" consists of carbohydrates (not serving primarily as carbon sources) that permit cells to respond to other growth substances; prominent in this class are the hexitols myo-inositol, scyllo-inositol, and sorbitol. b) "Active component" contains, in part, the auxins and the kinins.

In personal communication, Steward and Pollard pointed out the similarity in the behavior of hexitols and exogenous auxin: both interact synergistically with the kinin fraction in the growth of carrot phloem explants. They suggested that the carrot tissue might be using the hexitols to produce indoleacetic acid (IAA) from tryptophan. This suggestion was based on a newly described pathway for auxin biosynthesis, in which phenolic ortho-diols form oxidants which deaminate and decarboxylate tryptophan to yield the auxin IAA.⁽²⁾ Accordingly, we have determined whether hexitols, and also hexoses, can substitute for the phenols in the above conversion of tryptophan to auxin.

Following procedures described elsewhere,⁽²⁾ fresh preparations of the auxin-forming enzyme were made from carrot phloem tissues, *Coleus* terminal buds, leaves and buds of the mung bean seedling, etiolated *Avena* coleoptiles, and the livers of 6-week-old CF No. 1 female mice. Another enzyme preparation was "salted out" from a neutral 0.1 M phosphate dispersion of carrot phloem tissue with ammonium sulfate at 0.7 saturation. These preparations were incubated with L-tryptophan under conditions of substrate saturation and linear rates of IAA formation.⁽²⁾ The mixture was heated to halt the reaction and then fractionated by extraction with diethyl ether at pH 8 and 3. Aliquots of the ether-soluble acidic products were assayed for auxin activity by the coleoptile section test.⁽³⁾ Additional aliquots were subjected to ascending chromatography on Whatman No. 1 paper with a solvent system consisting of methanol/*n*-butanol/0.1 N acetic acid (1:1:98 v/v/v) (cf. Reference 4). The dried chromatogram was sectioned into 1/10 Rf units, each of which was assayed by the section test. This procedure separated the biological activity of IAA from that elicited by auxins with different migration rates.

The following hexoses and hexitols were tested at concentrations of 10^{-2} M in the incubation medium: D-sorbitol, m-inositol, D-glucose, fructose and sucrose. No auxin activity was observed when these compounds were incubated with tryptophan in the absence of enzyme.

Each enzyme preparation converted tryptophan to IAA. The addition of hexitol or hexose resulted in no significant enhancement. In some instances, glucose and fructose caused a reduction in the amount of auxin produced. There was no increase in auxin measured as unfractionated biological activity, as IAA, or as the summed total of the activities in the individual chromatogram segments. Comparable tests of the mung bean and Coleus enzymes with 10^{-2} M catechol resulted in over 9-fold enhancement of the amounts of IAA produced.

When 1 ml 10% ZnSO_4 followed by 1 ml 0.63 N NaOH was used instead of heat to stop enzyme activity at the end of the incubation period, considerable auxin activity was noted at the origin at the chromatogram in addition to that at the R_f of IAA (0.6 - 0.7). The addition of the carbohydrates to the media did not increase significantly the amount of either auxin.

It may be inferred that the hexitols interact with kinin-like factors in some manner other than by supplying auxin. How these alcohols function in growth remains obscure. The carbohydrates tested contain vicinal hydroxy groups on cyclohexane, pyranose, furanose and aliphatic carbon skeletons. Their inactivity in the conversion of tryptophan to auxin suggests that a carbocyclic as well as unsaturated ring structure is required for formation of the requisite oxidant.

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FURTHER ASPECTS OF THE PHOTOPERIODIC RESPONSES
OF SEQUOIA GIGANTEA

John Skok

It has been shown that Sequoia gigantea seedlings are markedly responsive to photoperiod.⁽¹⁾ Further experiments were, therefore, carried out to determine in more detail the kind of control mechanisms that may be involved.

Seedlings were grown on both long and short photoperiods. Others were grown under regimes in which the long dark periods were interrupted with short light breaks, or in which the long light periods were interrupted either with 1-hr dark periods or with 1-hr low-intensity light periods.

The results of the experiments in which the dark period was interrupted indicated that the stimulatory effects on growth of long photoperiods and the depressive effects of short photoperiods operate through a dark-dependent mechanism. The short-day effects were largely nullified by the daily interruptions of the long dark period with short light breaks.

The results obtained in the light period interruption experiments were somewhat more difficult to interpret, but they were not inconsistent with the above conclusion that the photoperiodic responses are based on a dark-dependent mechanism. Significant increases in certain growth measurements were attained in plants growing on long photoperiods when the long light period was interrupted with daily 1-hr periods of either darkness (dark-interrupted) or low-intensity (low-intensity-interrupted) light. If decreases had been obtained in the dark-interrupted lot, a light-dependent mechanism would be indicated. Under a dark-dependent mechanism, no effects would normally be expected. The observed increases, plus the results obtained with the low-intensity-interrupted lots serve to emphasize the general complexity of the photoperiodic mechanisms in woody species.

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SPECTRAL RESPONSE OF PHOSPHATE METABOLISM IN GERMINATING LETTUCE SEEDS

Kenneth Surrey

The requirement of visible light for lettuce seed germination was first demonstrated by Flint and McAlister.⁽¹⁾ Later, the Beltsville group^(2,3) established that the active region was the red end of the visible spectrum and that the activation caused by red light could be nullified repeatedly by the far-red radiation.

In any biological activity the expenditure of energy is a prerequisite for morphological expression (e.g. cell expansion, division, differentiation, dedifferentiation, etc.). In seed germination the energy is supplied from the various metabolites stored in the cotyledons. Since phosphate intermediates are known to be involved in various energy-transferring processes, the activation of phosphate metabolism must precede the morphological outcome (root initiation) in the seed germination.

In view of the effect of light and the endogenous energy requirements of the seed, the present study was conducted to observe the effects of monochromatic light on phosphate metabolism and to explore its possible role in the initiation of the photoactivated germination process. The results are expressed in terms of uptake of P^{32} -orthophosphate (Pi), acid-soluble organic phosphates (Po) and P^{32} -labeled phospholipid (Pl) fractions.

Methods and Materials

Each sample consisted of 50 lettuce seeds (Lactuca sativa L. var. Grand Rapids), placed on a filter paper in a 3-cm petri dish to which 2 ml of P^{32} -orthophosphate solution (about $3 \mu\text{c/ml}$) was later added. Unless otherwise stated the wet seeds were kept in the dark for 12 hr before they were subjected to various spectral treatments. The biological spectrograph⁽⁴⁾ was used for shorter exposures and monochromators fitted with interference filters^(5,6) were used for prolonged red, far-red radiation. At a desired stage of germination the seeds were thoroughly washed with water and homogenized in 4.7 ml of aqueous phase (5 g TCA, 10 g silicotungstic acid, and 1 g ammonium molybdate per 100 ml H_2O), and 5.3 ml of organic phase (isobutanol). Both aqueous and organic phases were modified from Ernster et al.⁽⁷⁾ to achieve the maximum recovery of Pi, to facilitate Pl extraction and to minimize the hydrolysis of acid-labile organic phosphates. The homogenate was transferred to a centrifuge tube, agitated vigorously and then centrifuged. Equal aliquots from each phase (0.1 ml, representing one seed) were taken for radioactivity determinations.

The aqueous phase extracted acid-soluble Po, whereas the organic phase contained Pi (as phosphomolybdate) and alcohol-soluble Pl. The organic phase extract was chromatographed one-dimensionally in acetone-TCA solvent to separate Pi and Pl, and the developed strips were scanned radiometrically⁽⁸⁾ to determine the radioactivity of each component (see Figure 43).

Figure 43

Radiometric scan of chromatogram of organic phase extract of lettuce seedlings developed in acetone-TCA solvent, showing the resolution of phospholipids and phosphomolybdate complex. The dotted curve, obtained by chromatographing p^{32} -orthophosphate separately, is superimposed to indicate its relative position.

For the identification of phospholipids, seedlings were extracted at different stages of germination directly with ethanol, isobutanol or acetone. These extracts, along with the organic phase extract, were chromatographed two-dimensionally,⁽⁹⁾ after which the individual chromatograms were autoradiographed. In each of the finished radiograms a prominent dense spot was located close to the solvent front; this corresponded to lecithins and phosphatidyl glycerols, with subjacent areas approximating the migration of phosphatidyl inositols.⁽⁹⁾ A similar examination of the aqueous phase extract and of the authentic samples indicate that it was apparently composed mainly of sugar phosphates and free nucleotides.

Results

Responses of phosphate metabolism under continuous red and far-red irradiation. Changes in phosphate utilization of lettuce seeds germinated under continuous red, under far-red, and in the dark are shown in Figure 44. It can be seen that during the initial 12 hr the seeds did not assimilate phosphate to any great extent. Thereafter, their activities are controlled by the respective light bands. For example, red light enhanced total as well as fractional phosphate utilization, while far-red suppressed; the dark-grown seeds retained their intermediate activity. Differences can also be seen in the time at which each group attained its respective maximal levels. As shown in the figure, Po formation in red, dark and far-red reaches the maximum at about 24, 40 and 56 hr, respectively, from the beginning of imbibition. This is followed by a steady decline in the red so that at about 52 hr Pi exceeds the Po level. In the unirradiated seeds a

crossover between Pi and Po comes at about 64 hr, and in the far-red there is no indication of any reversal. Similar differences can also be seen in the P_I fractions: the activities under red, dark and far-red reach their maximal levels at about 28, 36, and 44 hr, respectively.

Figure 44. Phosphate metabolic response (Pi, Po and P_I) of lettuce seeds germinated under continuous red light and far-red light and in darkness. Incident energies of both red and far-red bands were about 10 ergs/mm²/sec.

Influences of monochromatic light on metabolism. Phosphate utilization of the hydrated seeds as affected by monochromatic light of various wavelengths is depicted in Figure 45. As is evident from the figure (top), both Pi and Po show a considerable enhancement between 550 and 650 m μ with a decline on either side of this potentiating band. Beyond 700 m μ in the far-red region, the activities level off, whereas towards the shorter wavelengths the phosphate levels after declining in the blue region (475 m μ), once again rise in the violet light (400 m μ). The latter rise is then followed by a second drop so that the phosphate levels now fall (at 350 m μ) slightly below the dark control and remain at the lowest state through the remaining ultraviolet regions. It may be noted that throughout the visible spectrum Po remains consistently higher than Pi, whereas in the ultraviolet the distinction between the two fractions is obviated.

To observe the effect of an intervening dark period between irradiation and the time of harvest, another similar experiment was tried, in which the seedlings were analyzed at 64 hr from the beginning of imbibition. The results (Figure 45, bottom) show a photoresponse parallel to that illustrated in the top part of the same figure. The main differences lie in the reversal of Pi and Po levels over the potentiating region and the elevation of Po with respect to Pi in the ultraviolet spectrum, caused apparently by an extension of the germination period.

Figure 45. (Top). Phosphate metabolic response of dark-imbibed lettuce seeds exposed to various wavelengths of the visible and ultraviolet spectra for 3 hr and analyzed at 36 hr from the beginning of imbibition. P_0 in this figure represents acid-soluble organic phosphate plus phospholipids. P_i , as usual, was derived from phosphomolybdate complex. Average incident energies at each sampling position of the focal curve was about 7 ergs/mm²/sec.

(Bottom). Same procedure as in the top graph except that the seeds were processed at 64 hr from the beginning of imbibition.

Photoreversible metabolic responses. The previous section presents evidence for the existence of a potentiating region extending from 550 to 650 m μ . To determine whether the induced metabolism in this region can be suppressed by far-red (750 m μ) light, three loci (550, 600 and 650 m μ) were arbitrarily selected. Figure 46 (top) shows the repeated reversals when the seeds were exposed initially to 750 m μ , then to an alternating sequence consisting of one of the potentiating wavelengths and 750 m μ . The lower part of the same figure shows the reverse alternating sequence. Here, one batch was initially exposed to 650, the second to 600 and the third to 550 m μ light. Each batch was then alternated between 750 m μ and the locus of its initial exposure. The figure shows that although there are slight differences among the induced responses at 550,

600 and 650 $m\mu$, and similar variations among the suppressed activities at 750 $m\mu$, nonetheless, an over-all reversibility is clear. It therefore indicates that the enhancing effect of any one wavelength within the potentiating spectral band can be repeatedly suppressed by the far-red light, and vice versa. Secondly, it is evident that metabolism is affected solely by the wavelength of the terminating exposure and not by that of the initial or any other subsequent exposure.

Figure 46. (Top). Repeated reversals of phosphate metabolic responses (P_i , P_o , and P_l) of three batches of hydrated lettuce seeds exposed initially to 750 $m\mu$ locus. One batch (left) was then alternated between 650 and 750, the second (middle) between 600 and 750, and the third batch (right) between 550 and 750 $m\mu$. Each exposure lasted 10 min and was accompanied by removal of one sample from each group to the dark. Fifty-six hours from the beginning of imbibition seeds were analyzed for phosphate distribution. Incident energies at 750, 650, 600 and 550 $m\mu$ positions were approximately 16, 8, 16, and 48 $\text{ergs/mm}^2/\text{sec}$ respectively.

(Bottom). Same as above with the exception that the initial radiation sequence was reversed, i. e., one batch was initially exposed to 650, second to 600 and third to 550 $m\mu$ locus. Each batch was then alternated between 750 $m\mu$ and the wavelength of its initial exposure.

Discussion

Spectral responses of phosphate utilization (Figure 45; cf. Ref. 10) are comparable to that of germination.^(1,2,10) If however, a series of similar metabolic studies is conducted in which the time of irradiation is

decreased for each successive determination, eventually a sharp peak is obtained at $650\text{ m}\mu$. The effectiveness decreased from the red end of the spectrum towards the orange, yellow and green lights, and an observed peak in the violet (Figure 45) was also eliminated.

Increasing the irradiance of a single exposure from 3 to 8 hr did not alter the metabolic response. Also, no significant change was introduced when the seeds were given three intermittent exposures of 8 hr, each interposed by 16 hr of darkness.⁽¹⁰⁾ This indicates that a single 3-hr exposure was sufficient to ensure a maximal response.

Not only germination but also phosphate metabolism is reversibly affected by the red and the far-red light bands.⁽¹⁰⁾ In addition to the red, far-red reversible reaction, the existence of a similar phenomenon, operating for orange as well as green light each against far-red is now presented (Figure 46). A complete reversion was obtained only when the radiant energies of the two counteracting wavelengths were closely regulated. The energy relationship for each antagonistic pair is given in Figure 46.

Formation of chlorophyll and its participation in inducing phosphate metabolic activity under continuous red light did not seem to be a decisive factor. This was substantiated by exposing the imbibed seeds to red light for only 10 min and then allowed them to develop in the dark. Periodic sampling of these seedlings indicated a response similar to that shown in Figure 44 "red." The observation is in accord with that of Borthwick et al.⁽¹¹⁾ where a similar role of chlorophyll in seed germination was ruled out.

Acetyl-S-CoA is implicated in the promotion of the conversion of lipids into starch in the photosensitized germination process.⁽¹²⁾ Incorporation of radiophosphate into lipid constituents indicates that Pl formation also occurs during germination and can be detected as early as that of Pi and Po. The fact that phospholipid metabolism exhibits a photoreaction analogous to those of Pi and Po (Figures 44 and 46), implies that light does not effect Pl formation selectively; hence the entire phosphate metabolic response is geared through a single photoreactive system - the bottle-neck (concept of Krebs and Kornberg discussed in Reference 12).

The appearance of a radicle is not a prerequisite to activate phosphate metabolism. Seeds under continuous or intermittent far-red exposures showed no visible signs of radicle protrusion yet they attained a capacity to utilize phosphate in measurable quantities (Figures 44 and 46).

It appears then that far-red light does not completely suppress the initiation of phosphate metabolic processes of the imbibed seeds, which consist mostly of cotyledonous tissue. Instead, it may interfere with the

mitotic activities of the embryonic cells. The impairment in nuclear functions may be the determining factor caused by the lack of ATP, an impairment which can be remedied when the far-red-inhibited seeds are exposed to the red end of the visible spectrum (Figure 46). The suggestion is consistent with that of Wolff⁽¹³⁾ who observed that far-red, which acts as an inhibitor of various morphogenetic processes, also acts as an inhibitor of cell division. Furthermore, red, far-red dependence of mitochondria for the generation of ATP,⁽¹⁴⁾ and the association of phytochrome with isolated organelles,⁽¹⁵⁾ furnish additional evidence that photoresponse could be channeled, in vivo, through the mitochondrial-phytochrome system.

Summary

The photoresponse of lettuce seed germination is shown to parallel its phosphate metabolic activity: a) When seeds were continuously irradiated, red light accelerated and far-red suppressed their phosphate uptake and esterification activities; b) the influence of monochromatic light on phosphate metabolism of seeds, determined after 36 and 64 hr of germination, respectively, indicated maximum potentiation between 550 and 650 m μ , maximum suppression beyond 700 m μ and a partial suppression at 475 m μ . Stimulation encountered at 400 m μ was further suppressed by the shorter wavelengths of the ultraviolet spectrum; c) photoactivation of phosphate metabolism by the three loci (550, 600 and 650 m μ) of the potentiating spectral band was found to be reversed repeatedly by the far-red (750 m μ) light, and vice versa. Complete reversibility depended entirely upon the magnitudes of the radiant flux for the two counteracting wavelengths, and this was characteristic for each antagonistic pair. In view of the presence of phytochrome in or around the isolated mitochondria and the photosensitive nature of mitochondrial phosphorylation, it is suggested that a part of the energy flow required for cellular development may be channeled through the mitochondrial-phytochrome system.

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GAMMA-RAY SPECTROSCOPY IN THE UPPER ATMOSPHERE

Philip F. Gustafson

A γ -ray detector and associated electronic circuitry that may be carried aloft by a moderate size (6000 g) weather balloon has been designed and built by the Electronics Division at Argonne. The detector is a $\text{CaI}(\text{Tl})$ crystal, $1\frac{1}{2}$ in. in diameter and $1\frac{3}{4}$ in. long, enclosed in $\frac{1}{8}$ in. Pilot B plastic to make a phoswitch for charged particle rejection, coupled to an RCA 6199 photomultiplier tube. Pulses from the CaI portion of the phoswitch pass through a normally open linear gate into a pulse amplitude-to-time converter. If a pulse from the Pilot B portion of the phoswitch occurs in coincidence with a CaI pulse the linear gate closes, and no information from this event reaches the converter. The converter output pulse is used to turn off the carrier of a conventional 1680 Mc radiosonde transmitter. The entire balloon-borne package including batteries weighs less than 12 pounds.

The transmitted signal is received by a ground-based GMD2 radio receiver and tracking antenna, the 1680 Mc signal being interrupted for time intervals which are proportional to the height of the CaI pulse. The interrupted carrier is fed into a time-to-height converter and thence into a count-rate meter and a multichannel analyzer for conventional pulse analysis.

Figure 47. Na^{22} calibration spectrum, Flight #4.

By means of this balloon-ground station system it has been possible to obtain γ -ray spectra of air-borne radioactivity in situ. The count-rate recorder permits a continuous measure of gross γ count-rate as a function of altitude. The energy region observed ranged from 0.25 to 4.0 Mev. Pulse analysis was done using an RIDL 200-channel analyzer, photographing the visual display periodically, with total readout by IBM typewriter at the end of a run. Energy calibrations (Figure 47) were made using a Na^{22} source.

The first detector was flown successfully from the Weather Station at Chanute Air Force Base, Rantoul, Illinois on November 4, 1961. A Na^{22} source was included in the balloon-borne package; subtraction of the Na^{22} spectrum showed a spectrum due to fission debris attributed to the recently concluded Soviet tests. Unfortunately the balloon burst at about 42,000 feet, and hence data only up to the low stratosphere were obtained.

Since the over-all operation of the system was encouraging, additional units were built and 6 of these were flown at Fort Churchill, Manitoba, Canada during the latter part of November as a joint USAEC-U.S. Weather

Bureau project with the cooperation of the Canadian Weather Service. Continued difficulties were experienced with the balloons due to freezing and icing in the vicinity of 30,000 - 40,000 feet, where the temperature dropped to -50 to -65°C . A successful flight was made on November 26, reaching an altitude of 120,000 feet. Although a temporary electronic failure precluded data collection between 75,000 and 90,000 feet, much valuable information was obtained, including a measure of gross γ -count-rate versus altitude as shown in Figure 48. The rapid increase in count-rate with altitude is due primarily to γ -rays produced by cosmic ray interactions with the atmosphere; such interactions reach a broad maximum at 55,000 feet. Additional γ -radiation was encountered coming from nuclear test debris throughout the troposphere (tropopause height - 28,000 feet), with intense, discrete, layers of fission-product activity discernible at 40,000 - 45,000 and 62,000 - 65,000 feet. The net γ -spectrum in the latter layer is shown in Figure 49, and indicates the presence of Zr^{95} - Nb^{95} , Ba^{140} - La^{140} , $\text{Ru}^{103,106}$, and other fission nuclides which emit γ -rays in the region of 0.511 Mev. A line due to the annihilation of cosmic ray-produced positrons also occurs at this energy, but correction for this is subject to considerable uncertainty at this time. The spectrum seen above 97,000 feet to the top of the flight, Figure 50, indicates a single line at 0.511 Mev arising from annihilation, and a continuum extending to 3.8 Mev. No lines due to fission products were observed in this altitude range, which implies the absence of appreciable amounts of fission debris above balloon altitude (theoretically up to at least 150,000 feet) since the γ -rays from such activity would reach the 100,000 to 120,000-foot level with only slight attenuation in the intervening, rarefied atmosphere.

Figure 48. Gross count-rate versus altitude, Flight #4.
(Energy range 0.25 to 3.8 Mev)

Figure 49. Net γ -ray spectrum
62,000 - 70,000 feet.
Flight #4.

Figure 50. Net γ -ray spectrum
97,000 - 120,000 feet.
Flight #4.

The other five flights, although not reaching high altitude, showed day-to-day differences in the concentration of fission products in the troposphere, and the first flight (Nov. 24) showed an intense layer of fission activity at the tropopause (27,000 feet).

The balloon γ -ray spectrometer has proven its usefulness as a means of measuring both intensity and composition of atmospheric radioactivity. Further flights, pending the availability of appropriate balloons, are contemplated. Such flights will be directed primarily toward a search for high-altitude polar debris and in following the descent and southward movement of such debris during the spring of 1962.

It is a pleasure to acknowledge the fine work and assistance of the Electronics Division in this endeavor. Particular recognition must go to Dr. John Gilroy (Director), Bob Epstein, Mike Strauss, Dick Keener, Ed Johnson, and Joe Haumann of Electronics and to Hal Clark of the U. S. Weather Bureau, all of whom had the dubious pleasure of spending Thanksgiving together in the Canadian wilderness.

P^{32} UPTAKE AS AN INDICATOR OF TRITIATED THYMIDINE TOXICITY

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Observations of toxicity of tritiated thymidine (H^3 -thymidine) secondary to its selective intranuclear incorporation into DNA and the release of its energy of disintegration within the nucleus has led to concern over the hazards involved in using tritium as a deoxyribonucleic acid label in man.⁽¹⁻³⁾ This is especially true since it has been reported that at a dose level of 1 $\mu\text{c/g}$ tritiated thymidine is a carcinogen in mice.⁽⁴⁾ This lends weight to the existing evidence that tritiated thymidine can produce significant changes in mammalian cells.⁽⁵⁻⁸⁾

It has been known for a long time that ionizing radiations produce gene mutations, chromosome damage, inhibition of mitosis, and cell death. The decreased uptake of phosphate following exposure to X-rays that has been noted⁽⁹⁻¹³⁾ suggested that P^{32} was incorporated directly into the nuclear material and that uptake was thus proportional to mitotic activity. Since H^3 -thymidine exerts a selective effect on mitotic activity through its incorporation in DNA, it seemed reasonable to determine whether this specific internal irradiation might have an inhibiting effect on P^{32} uptake similar to that produced by external radiation. This preliminary report describes a study of P^{32} uptake and tissue distribution as related to prior administration of H^3 -thymidine.

Materials and Methods

Eighty-four CF No. 1 male mice, 5-6 weeks old, weighing 15-20 g, were injected intraperitoneally at dose levels of 1, 20 or 100 μc of H^3 -thymidine/g body weight. The specific activity of the thymidine was 0.36 c/mM (Schwarz BioResearch, Inc.). The animals at each dose level were then randomly divided into two groups. The first group received 0.15 $\mu\text{c } P^{32}/\text{g}$, given intraperitoneally 1 day following the injection of H^3 -thymidine. The second group received 0.15 $\mu\text{c } P^{32}/\text{g}$, intraperitoneally 7 days following the injection of H^3 -thymidine. Randomly paired controls received P^{32} without prior injection of H^3 -thymidine. All animals were given food and water ad libitum. Animals were sacrificed at intervals of 20, 40, 60 and 90 min, 2, 4, 24 and 48 hr, and 8 days following injection of P^{32} .

In most cases at least 2 animals were killed at each interval, for each group at all dose levels. Blood samples were obtained by severing the carotid artery and collecting the blood in tared, No. 1 porcelain, Coors capsules. From 0.5 to 1.0 g of blood per mouse was obtained by this method.

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The mice were dissected into the following portions: liver, spleen, kidneys, testis, viscera (all the remaining contents of the abdomen and thorax), femur, and carcass (remainder of the mouse, principally muscle, pelt, and skeleton). The organs were isolated, weighed and ashed in Coors dishes, and the P^{32} activity was determined with a thin-window GM counter.

Results

Special attention was given to the testis and spleen, considered among the most radiation-sensitive organs. In Figure 51 is plotted the % of the P^{32} retained per organ versus time after injection for the testis, spleen, and kidneys, combining all experimental groups. No differences either in the rate or in the absolute amount of uptake of P^{32} were seen between the controls and experimental group. This was also true for the other tissues that were assayed.

Figure 51. Effect of tritiated thymidine on the uptake of P^{32} in CF No. 1 male mice.

Discussion

The failure to demonstrate inhibition or alteration of P^{32} uptake following H^3 -thymidine administration may be due to several factors. First, intranuclear incorporation of H^3 -thymidine following a single injection is probably limited to the first half-hour. Only those cells actually synthesizing DNA during this brief period would incorporate H^3 -thymidine and subsequently be influenced by it.

Second, it is not certain that the doses of H^3 -thymidine used compare with the doses of X-rays required to inhibit DNA synthesis. It has been

suggested⁽¹⁴⁾ that 1 μ c of H³-thymidine/g body weight is equivalent to 2-5 r of X-rays, and in the testis after as little as 10 r of external irradiation, effects can be detected. However, atrophy of the testis proceeds slowly, taking from 3-4 weeks, and this is longer than the 16-hr and 7-day intervals between the injection of H³-thymidine and sacrifice in our experiments.

Third, the fraction of P³² uptake entering into DNA synthesis may be very small compared with the uptake of phosphate into bone mineral, energy-rich compounds and other phosphorylated compounds, so that significant alteration in the rate or amount of DNA synthesis may not be apparent in the over-all uptake of P³². P³² not used for DNA synthesis may be shunted immediately to other pathways. Extraction of DNA from the tissues, with hydrolysis and separation of the constituent bases and determination of their activities might possibly have revealed differential P³² uptake into DNA following H³-thymidine.

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