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**BIOLOGICAL AND MEDICAL
RESEARCH DIVISION**

SEMIANNUAL REPORT

July through December, 1959

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SEMIANNUAL REPORT

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ILLINOIS BONE TUMOR DEATH CERTIFICATE STUDY

Harry Auerbach

Several epidemiological studies are being conducted to study the possible relationship between environmental Ra^{226} and incidence of bone tumors. Values for the incidence of such tumors in humans are difficult to secure without large-scale, carefully conducted surveys to ensure that the figures being used are true bone tumor rates. Data on bone tumor deaths are, however, readily available in great detail from State Bureaus of Vital Statistics.

In the course of the collection of such data for the calculation of bone tumor death rates for Illinois, it became apparent that a surprisingly high percentage of all bone tumor deaths as reported on death certificates (among white males, approximately 35%) were allocated to jaw bone tumors. Review of pathology texts and discussions with Illinois pathologists tended to dispute this finding. Direct examination of the death certificates led to the conclusion that a large artefact probably existed in the registration of bone tumor deaths. In addition to the apparent large numbers of jaw bone tumors being reported, the use of ambiguous and inexact terminology tended to cast doubt on the accuracy of present registration of bone tumor deaths. The validity of all the epidemiological studies related to environmental Ra^{226} and bone tumor death rests on the determination of exact figures of true bone tumor deaths.

A study was, therefore, undertaken to determine the true bone tumor death rate in Illinois. All bone tumor deaths in Illinois for the period January 1, 1958 to June 30, 1959, as reported on the death certificates, were studied. With the cooperation of the Illinois Department of Public Health and its Bureau of Vital Statistics and by contract with the National Office of Vital Statistics of the Public Health Service, a careful study was made of each bone tumor death reported.

A questionnaire was sent to each certifying physician within two months of the date he signed a death certificate listing bone tumor as the cause of death. This questionnaire elicited the methods of diagnosis used, the histopathological findings, the certitude of diagnosis, and a variety of other information designed to permit an estimate of the exact cause of death. Each physician was also requested to refer the names of all other physicians participating in the diagnosis or the treatment of the patient. These referred physicians were then sent similar questionnaires for evaluation. The family informants supplying the personal data on the death certificates were also sent questionnaires to elicit the names of any other physicians to be approached.

When the questionnaires were returned, they were coded and evaluated in the following manner. Where a physician reported a clear-cut histopathological diagnosis based on biopsy, surgical specimen, or autopsy protocol, this was coded directly as evaluated. All other questionnaires were reviewed independently by a cancer clinician and a pathologist. On the basis of their review of all the material from each case, they evaluated the cases as (1) bone tumor, (2) not bone tumor, (3) probably bone tumor, or (4) probably not bone tumor.

In the 18-month period from January 1, 1958 to June 30, 1959, 166 deaths due to bone tumor were recorded in Illinois. Questionnaires were returned from 158 certifying physicians, an initial return rate of 95.2%. With the other sources approached, questionnaires were finally received for 165 of the 166 cases, a return rate of 99.4%.

In a preliminary analysis based on the evaluation of all questionnaires returned, only 34.3% of all cases were considered to be true bone tumors, and an additional 1.2% were considered to be "probably true bone tumor." Half of all cases (50.6%) were held to be definitely not bone tumors and an additional 6.0% were considered to be "probably not true bone tumor." An additional 4.8% were considered to be malignant neoplasms, but no definite decision could be reached as to the exact type. In 2.4% of the cases, the physicians evaluating were uncertain as to whether a malignancy in fact existed, and in 0.6% or 1 case it was felt that a benign neoplasm existed.

These findings indicate that in the registration of deaths due to bone tumors there is an artefact of almost 65%, and presently published death rates associated with this cause are highly overstated. This condition is not due to anything peculiar to Illinois, but exists because of the National Coding rules, which are followed in all of the United States and all countries using the International Causes of Death Coding System.

An enlarged study has now been started to examine the registration of bone tumor death certificates in seven other states of the United States; this, together with the present data from Illinois, will permit the analysis of almost 40% of all bone tumor deaths in this country in one year. Such analysis will permit the formulation of changes to be made in coding rules to prevent the over-registration of bone tumors and to provide exact figures for use in epidemiological studies now in progress. It will also give a true base-line figure of bone tumor deaths so that future studies may be performed to detect possible increases arising from environmental causes.

THE TOTAL TUMOR INCIDENCE IN LAF₁ MICE EXPOSED TO CHRONIC GAMMA IRRADIATION

S. Lesher, G. A. Sacher, and Douglas Grahn*

The development of tumors is one of the principal causes of death in the long-term survivors of mice exposed to chronic irradiation. The incidence of tumors was determined in LAF₁ mice exposed to low-dose, daily, γ -irradiation at dose levels ranging from zero to 56 r per day. The mice were allowed to die naturally, and all organs were examined carefully for tumors. Tissues were also studied histologically to verify diagnosis. The experimental design and the gamma facilities were described in a previous report.⁽¹⁾

The total tumor incidence is shown in Table 1 (males) and Table 2 (females). The number and variety of tumors are much greater in the females. The incidence of most tumors increases at 6 r and 12 r per day but decreases rapidly above this level. In tumors that have a tendency to develop late in life (e.g., liver, skin, bone) incidence and latent period are not affected by irradiation. However, there is a noticeable reduction in the pretumorous period for thymic lymphomas and ovarian, pulmonary and mammary tumors.

TABLE 1

Tumor incidence in male LAF₁ mice exposed to daily Co⁶⁰ γ -irradiation

Dose, r/day	Number of mice	Number of tumors					
		Lymphoid	Pulmonary	Hepatomas	Skin	Intestinal	Misc.
0	266	21	25	8	1		1
6	120	9	12	7			2
12	183	19	13	6			
24	183	31	6	2		1	
32	150	24	4				
43	150	21	1				
49	135	9					
56	120	3	1				

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TABLE 2

Tumor incidence in female LAF₁ mice exposed
to daily Co⁶⁰ γ -irradiation

Dose, r/day.	Number of mice	Number of tumors								
		Lymphoid	Ovarian	Pulmonary	Mammary	Hepatomas	Skin	Bone	Intestinal	Misc.
0	262	50	41	26	8	17	5	2	1	3
6	120	31	35	19	10	5	3	1	1	2
12	183	46	31	18	10	5	6	1	1	1
24	183	56	6	5	4		3	2		
32	150	48	1	2	1		1		1	
43	150	34		2						
49	135	17		1						
56	120	7								

In the female mice the number with more than one primary tumor at death is high in the zero, 6-r and 12-r groups (Table 3).

TABLE 3

Number of mice with tumors at death, total number of tumors,
and number of mice with 2 or more tumors

	Dose, r/day							
	0	6	12	24	32	43	49	56
Males								
No. of mice with tumors	48	27	35	39	27	22	9	4
Percent with tumors	18	23	19	21	19	15	7	3
Total No. tumors	56	30	38	40	28	22	9	4
No. mice with 2 or more tumor types	6	3	3	1	1			
Females								
No. of mice with tumors	117	65	99	76	51	36	18	8
Percent with tumors	45	54	54	38	34	24	13	7
Total No. tumors	158	105	118	76	54	37	18	8
No. mice with 2 or more tumor types	32	32	15	3	2	1		

Reference

1. Sacher, G. A., D. Grahn, S. Leshner, and K. Hamilton. The Gamma Ray Toxicity Program: Calibration and equipment. Report on Biological, Medical and Biophysics Programs, Argonne National Laboratory. ANL-5378 (1955), pp. 91-94.

THE EFFECTS OF CHRONIC GAMMA IRRADIATION ON MOUSE TESTIS

B. R. Nebel

A part of the Gamma Ray Toxicity Program is an experiment in which CAF₁ mice are exposed continuously to Co⁶⁰ γ -rays at varying dose rates.⁽¹⁾ The present study involves histocytological and other examination of the testes of the exposed mice and statistical evaluation of the results. This report summarizes observations at four dose rates: 12, 24, 43, and 74 r/day.

The general pattern of the spermatogenic response was obtained by Chalkley counts in which the categories individually recorded were Sertoli cells, spermatogonia, early and late spermatocytes, spermatids, spermatozoa, space inside the tubules and vacant space outside the tubules. The results are summarized in Table 4 in which each value represents the average of 400 or more determinations. The control series consisted of 3 animals each for 4 age groups. These are lumped in Table 4, since the static picture of the sections from these age groups showed no significant changes with age. We do not, however, know what cell turnover or rate of development would be if each age group were studied with tritiated thymidine.⁽²⁾

Since the Chalkley method is unreliable for cells present in low frequency, direct counts were made on the Type A and intermediate spermatogonia as well as the Type B spermatogonia and leptotene spermatocytes. Also, pycnotic cells, which in this material were rare, were separately recorded. These direct observations are discussed separately.

Discussion of Chalkley Counts

Sertoli cells. Inspection of the data shows that the percentage of Sertoli cell number increase is consistent within each of the four series. It is largest in the 12-r series, i.e., 7.05 times the control value (Table 5). The apparent increase in Sertoli cells is primarily due to tubular contraction. As suggested earlier⁽³⁾ a decrease to one-half the tubular cross-sectional area corresponds to a doubling in the number of Sertoli cells. Table 5 shows that the factor of Sertoli cell increase is approximately equal to the reciprocal of the area (expressed as a fraction of control values). The values for decrease in tubular area corresponding to the last Chalkley count (e.g., 251-day for 12 r/day) in each of the series were taken from the fitted curves for tubular areas (see Figure 1). Inconsistencies in the relative positions of the 12- and 24-r/day curves of response appear rather regularly throughout our observations. In no case in these continuously irradiated mice did we observe blocked metaphases and restitution cells as described for single acute irradiations (1000 r).⁽³⁾

TABLE 4

Proportions of each cell type and of intratesticular and intratubular space obtained from statistical analysis of Chalkley counts. Values are expressed as percentages of total Chalkley "hits".

Dose rate, r/day	Exposure time, days	Cumulative dose r	N (Number of Chalkley "hits")	Sertoli cells	Spermatogonia (A, intermediate, B)	Early spermatocytes	Late spermatocytes	Total spermatocytes	Spermatids	Spermatozoa	Inner space	Outer space	Total space
Control	-	-	6000	2 60	2 45	18 69	1 74	20 43	18 62	10 95	29 58	15 39	44 95
12	1	12	1314	3 35	2 34	17 26	2 67	19 93	21 99	11 15	23 23	17 92	41 24
	8	96	1200	2 50	1 42	12 67	2 58	15 25	16 33	9 75	37 25	17 50	54 75
	18	216	1200	2 33	0 50	5 75	2 75	8 50	14 75	10 00	44 00	19 92	63 92
	25	300	1200	2 92	1 08	3 50	1 67	5 17	10 83	11 75	46 83	21 42	68 25
	63	756	1200	6 88	1 32	3 90	1 24	5 14	8 12	6 05	33 66	38 82	72 48
	91	1092	1200	7 75	0 50	2 00	0 58	2 58	4 00	3 08	42 08	40 00	82 08
	120	1140	1200	14 25	0 00	0 63	2 00	2 63	1 63	2 00	35 50	44 00	79 50
	152	1824	1200	18 67		0 08	0 33	0 42	0 00	0 00	38 08	42 83	80 91
	251	3012	1200	18 33		0 00	0 17	0 17			34 75	46 75	81 50
24	1	24	1200	3 47	2 77	13 44	6 79	20 23	21 83	14 63	19 63	17 45	37 08
	7	168	1200	2 33	0 67	12 33	5 50	17 83	22 08	14 92	24 83	17 33	42 16
	16	384	1200	2 58	0 08	1 58	2 17	3 75	23 33	17 42	29 42	23 42	52 84
	30	720	1200	7 33	0 00	0 17	0 08	0 25	1 08	16 17	25 00	50 17	75 17
	61	1464	1200	12 50		0 08	0 00	0 08	0 08	0 25	38 50	48 58	87 08
	134	3216	1200	13 67		0 00		0 00	0 00	0 00	35 00	51 33	86 33
43	1	43	1200	2 67	1 17	9 83	5 00	14 83	17 33	13 58	33 50	16 92	50 42
	7	301	1200	2 67	0 17	8 48	2 50	10 98	18 30	14 14	37 60	16 15	53 75
	20	860	1200	4 17	0 00	0 08	0 00	0 08	9 67	22 42	40 42	23 25	63 67
	28	1204	1200	8 00		0 00		0 00	1 00	17 00	43 00	31 00	74 00
	30	1290	1200	9 88					0 00	9 88	39 00	41 25	80 25
	45	1935	1200	16 33						0 00	48 08	35 58	83 66
	60	2580	1200	14 75							34 25	51 00	85 25
	61	2623	1200	12 50							37 75	49 75	87 50
74	1	74	1200	1 83	2 33	8 76	5 84	14 60	18 10	12 09	37 87	13 18	51 05
	3	222	1200	2 33	0 00	7 00	5 92	12 92	15 08	8 33	45 17	16 17	61 34
	9	666	1200	1 45		4 79	4 15	8 95	15 50	9 13	47 66	17 32	64 98
	15	1110	1200	2 08		0 00	0 00	0 00	17 42	11 33	43 50	25 67	69 17
	20	1480	1200	3 33					6 83	13 33	46 33	30 17	76 50
	36	2664	1200	10 25					0 00	0 33	51 33	38 08	89 41

TABLE 5

Relationship between (apparent) increase in Sertoli cell number and decrease in tubular cross-sectional area

Dose rate r/day	Tubule area after irradiation with 3000 r		Factor of Sertoli cell number increase
	% of control	Fraction of control	
12	12 06	1/8 29	7 05
24	11 35	1/8 81	5 26
43	19 86	1/5 04	4 81
74	26 95	1/3 71	3 94

Figure 1. Fitted curves for tubular area plotted against cumulative dose.

Spermatocytes. Counts on the total number of spermatocytes in the four series show very marked differences between the lower and the two higher intensities.

Normal development of spermatocytes requires slightly more than 9 days.⁽⁴⁾ Thus, it would be safe to assume that after the supply of Type B spermatogonia is cut off, normally developing spermatogonia would disappear, becoming spermatocytes. This seems essentially true for the 74- and 43-r/day series. Both of these series are compatible with the classical concept of water running out of a reservoir. The counts of the 12-r/day series indicate that there is considerable elongation of the life span of some spermatocytes. If we assume that the supply was cut off at 18 days or slightly earlier, the presence of spermatocytes at 120 and even 250 days indicates delayed development. At the same time the drastic reduction in percentage of spermatocytes observed on or after the 18th day suggests heavy progressive killing.

Spermatids and Spermatozoa. These cell categories show a higher radiation resistance.⁽⁵⁾ Thus, these cells reflect a cutoff in spermatocytes. This is true of all four series. Again the 12-r/day series shows delayed development of the spermatids and spermatozoa. It is notable that the lowest dose rate (12 r/day) shows the greatest effect on developmental rate.

Space. Inner space refers to the areas in sections inside the tubules not taken up by cells. Outer space refers to the areas outside the tubule but inside the limits of the testis. The data show that inner and outer space increase in the course of the experiment. The increase in inner space is due to cell death and removal. The increase in outer space is the result of tubular shrinkage.

Discussion of Direct Counts

Type A and intermediate spermatogonia. Direct spermatogonial counts were made in each of the four series using from 20 to 60 tubules for each point. The data are shown in Figure 2. In the 74- and 43-r/day series

Figure 2. Type A and intermediate spermatogonia per tubule after 4 radiation intensities. The survival of spermatogonia in the 12- and 24-r/day series appears identical and stands in marked contrast to the death of spermatogonia in the 43- and 74-r/day series.

no spermatogonia were found above 1100 and 2000 r respectively. In sharp contrast Type A and intermediate spermatogonia survive in the 12- and 24-r/day series. After an initial sharp drop these cells appear to adjust to the continuous irradiation. Apparently, spermatogonia are able to divide and survive in the intermediate and earlier state, and this mitotic increase is balanced by cell death as they develop into Type B spermatogonia. In Figure 3 a somewhat hypertrophic spermatogonium is shown after 15 days under 74 r/day (cumulative dose, 1110 r). Figure 4 shows spermatogonial division after 134 days under 24 r/day (cumulative dose, 3220 r). Figure 5 shows a group of three spermatogonial cells, one out of focus, one small, and one large, the latter in prophase, from the 12-r series at 419 days (cumulative dose, 5028 r).

It would be of interest to observe the genetic effects in spaced recovery experiments, inasmuch as the cytological observations suggest that there may be considerable selection at work during the prolonged survival of this cell category.

Type B spermatogonia and leptotene spermatocytes. Direct counts were made in each of the four series. Results are shown in Table 6. In the 12-r/day series a few Type B spermatogonia were still observed after 300 r (25 days) but were completely absent after that. Leptotenes followed the pattern closely. This compares well with the Chalkley data of the 12-r/day series. In the three series of higher intensity the disappearance of Type B spermatogonia and leptotene stages is much faster. Our figures show clearly that the leptotene is more resistant to the radiation than Type B spermatogonia, hence the disappearance of leptotenes is largely caused by progressive development. On the other hand, the high sensitivity of the Type B spermatogonia did not allow continued development of this cell stage in any of the four series. Thus, under chronic, as well as under acute irradiation, the Type B spermatogonial stage acts as a reversible block to temporarily prevent spermatogenesis.

Figure 3. A hypertrophic spermatogonium from the 74-r/day series after 15 days (cumulative dose, 1110 r).

Figure 4. Spermatogonial mitosis after 134 days of 24 r/day (cumulative dose, 3220 r). This is a metaphase in side view.

Figure 5

Spermatogonial cell in prophase in the 12-r/day series at 419 days (cumulative dose, 5028 r). Apparently two daughter cells both of which appear abnormal, lie beside the main cell.

TABLE 6

Direct counts of Type B spermatogonia
and leptotene spermatocytes

Dose rate, r/day	Day	Total dose, r	Type B spermatogonia	Leptotene spermatocytes
Control	100-971	0	14.2	54.4
12	1	12	12.5	52.9
	8	96	8.0	44.3
	18	216	2.9	9.3
	25	300	1.8	13.1
	65	780	0.0	4 tubules/100 contain some leptotene cells
24	1	24	7.5	51.1
	7	168	2.1	31.9
	16	384	0.0	0.0
43	1	43	5.7	51.3
	7	301	0.0	0.0
74	1	74	6.3	48.7
	3	222	1.4	56.5
	9	666	0.0	0.0

The figures listed above represent an average of 30 tubules in the case of the controls and an average of 15 tubules at each dose level in the treated series.

Pycnotic cells. The data from the direct counts of the pycnotic cells are summarized in Table 7. The incidence of pycnosis under chronic irradiation is low. This is due to the fact that the "life span" of a pycnotic cell is short.⁽⁶⁾ Even if one assumes that cell loss through pycnosis is about three times as high as our first-day figures (assuming an 8-hour "life span"), the loss would be of the order of one cell/tubule section/day. Estimating an average of 600 cells/tubule, a loss of one cell represents a decrease in population of 0.16%.

TABLE 7

Direct counts of pycnotic cells

Dose rate, r/day	Day after start of irradiation	Dose, r	Pycnotic cells/tubule
Control	200-658	0	0.10
12	1	12	0 14
	8	216	0 15
	25	300	0 18
	60	720	0 10
	91	1092	0 00
24	1	24	0 21
	7	168	0 19
	16	384	0 12
	30	720	0 09
	61	1464	0.00
43	1	43	0.34
	7	301	0 23
	20	860	0.11
	30	1290	0 14
	45	1935	0 00
74	1	74	0 36
	3	222	0 26
	9	666	0 13
	15	1110	0.08
	20	1480	0 02
	36	2664	0 00

Discussion of Testicular Measurements

In order to follow the spatial relationships within the testis under chronic exposures, measurements were made of tubular areas and testis areas. The trend of these measurements was compared with the trend of testis weights.

Testis weight. In chronic experiments testis weight loss follows the same two-component pattern described by Kohn and Kallman, (7) with an A component covering the first steep part of the curve and a B component covering the second less steep part. In our plots of testis weights vs. dosage (Figure 6) we fitted a line to our points in the four series using the formula

$$y = Ae^{-\alpha D} + Be^{-\beta D}$$

where $Ae^{-\alpha D}$ is the A component and $Be^{-\beta D}$ is the B component. A is anti-log a (intercept) for the difference plots (A); α is 2.303b, where b is the slope for the A component; D is the dosage (x); B is the antilog a for the B component; β is 2.303b for the B component. This is a preliminary attempt. We disregarded the first two points, where the weight is increased due to swelling. Kohn and Kallman do not mention the initial swelling which we have observed consistently.

Figure 6

Fitted curves for testis weights of the 4 dose-rate series plotted against cumulative dose.

Testis area. We also plotted the presently incomplete data for testis area (see Figure 7). "Total testis area" represents the direct measurements of the cross sections of the testis as they appear on the slides after sectioning. The decrease in testicular area followed the same pattern as loss in testicular weight. Preliminary curves were fitted as above. It appears that microscopic measurements of testis area as seen in section reflect the testis weight.

Figure 7

Fitted curves for testis area plotted against cumulative dose.

Tubule area. To obtain tubular area, over 3000 measurements were recorded. In each testicular cross section the ratio of spherical vs. elongate tubules was determined, and each category was evaluated by measuring the respective diameters. From these data the total area occupied by the tubules was calculated. Total tubule area was graphed in the same manner as testis weight and area (see Figure 1). The data for the 43- and 74-r/day series are not complete because the corresponding paraffin material has not yet been sectioned.

Table 8 shows the constants for the steep part of the curves for the three sets of data to be very similar. The divergence in the data of the 43- and 74-r/day series for testis and tubular areas is due to the fact that the points beyond 2700 r have not yet been evaluated.

TABLE 8

Values of α for the steep part of the curves for
testis weight, testis area, and tubular area*

Dose rate, r/day	α Testis weight	α Testis area	α Tubule area
12	-0.0034	-0.00188	-0.00151
24	-0.0042	-0.00289	-0.00319
43	-0.0018	-0.00059	-0.00060
74	-0.0014	-0.00037	-0.00057

*See page 18

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SURVIVAL OF GUINEA PIGS UNDER DURATION-OF-LIFE EXPOSURE TO GAMMA RAYS

G. A. Sacher and R. R. Robertson

Table 9 summarizes the results thus far of an experiment to determine the survival of guinea pigs under periodic exposure to $\text{Co}^{60}\gamma$ -rays. The weekly doses were delivered in a number of treatments, varying from 1 to 6, per week. The exposure pattern in Table 9 is given as a product in which the first term is the dose per treatment and the second term is the number of treatments given per week. Every treatment group spends part of its time in the control environment. In addition, control subgroups are transferred between two locations in patterns that match the movement patterns of the various exposure groups. These control groups are not separated in the table.

The actual times under treatment for the five replications are:

Replication	Days since entry
1	750
2	666
3	589
4	505
5	407

The mortality experience in control and exposed groups is compatible with the reports of previous investigations.

[illegible]

THE DENSITY OF MAMMALIAN TISSUES

N. A. Frigerio, H. E. Frigerio,* N. Bink, and R. Barhorst

A knowledge of tissue density is necessary for the computation of total dose and depth dose in organisms, as well as the dose to certain critical organs. In the absence of more precise information a value of 1.00 g/ml has customarily been employed. This serves as a fair approximation for whole organisms with large lung and peritoneal cavities but, since even blood and tissue fluids have minimum densities of the order of 1.04 g/ml,⁽¹⁾ it is a poor approximation for individual organs and homogeneous tissue regions.

Since the density enters into dose expressions in the exponent, small errors in density give rise to large errors in dose, particularly for the larger penetrations encountered in man and similar large mammals. This consideration, as well as the need for precise density values as guides in our formulations of true tissue equivalent substances for dosimetry, prompted us to determine the average densities of some of the more critical organs in a fairly typical mammal, the mouse.

Fifteen 6-month-old female CF No. 1 mice, weighing 28 to 32 g, were sacrificed. The organs were removed and weighed, first in air and then under distilled water, at temperatures between 24 and 25°C. Absolute densities were computed from the usual force considerations, and the IBM 610 computer was employed to effect corrections for temperature and density of the buoyant and supporting media.

Results are presented in Table 10. It may be seen that the average organ densities differ considerably among themselves and that each is markedly higher than 1.00 g/ml. Standard deviations and/or differences among individual animals are, however, quite small. Measurements on the organs of other mammals are currently in progress.

TABLE 10

Densities of mouse tissues

Organ tissue	Density at 25°C, g/ml
Brain	1.0475 $\pm$ 0.0009
Skeletal muscle	1.0583 $\pm$ 0.0012
Spleen	1.0826 $\pm$ 0.0011
Kidney	1.0547 $\pm$ 0.0008
Liver	1.0787 $\pm$ 0.0006

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LEWIS SALTS FOR USE IN ORGANOMETALLIC SYNTHESSES

N. A. Frigerio

In organometallic syntheses, anhydrous systems are employed to obviate undesirable aqueous side reactions. Since anhydrous forms of many of the more interesting inorganic salts are either unknown, difficult to synthesize, or unstable in air, organometallic syntheses are often reduced to circuitous routes or afford very poor yields. Thus, in the synthesis of metal phthalocyanines from dilithium phthalocyanine⁽¹⁾

the water of hydration drastically reduces the yield via such side reactions as

These hydrated salts may be regarded as Lewis salts⁽²⁾ formed by the filling of the electron-deficient orbitals of the Lewis acid with electrons derived from the Lewis base, water. This model predicts the possibility of displacing the water of hydration with a stronger Lewis base to yield a more stable and water-free Lewis salt. Dimethyl formamide [$\text{HCONMe}_2(\text{DMF})$] was selected for trial because it is both a strong Lewis base and an excellent solvent for organic and inorganic compounds.

Addition of DMF to $\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ gave immediate evidence of a mildly exothermic reaction, indicating that the free energy of formation of the uranyl nitrate-DMF compound is distinctly higher than that of the uranyl nitrate hexahydrate. Excess DMF was added to the system, and the product was recrystallized from DMF to constant melting point and composition, washed with acetone, and dried to constant weight at 1μ pressure. The yellow crystalline solid obtained melted at $177 \pm 0.3^\circ\text{C}$. Combustion analyses indicated a formula of $\text{UO}_2(\text{NO}_3)_2 \cdot 2\text{HCONMe}_2$.

	Percent			
	U	C	H	N
Calculated for $\text{UO}_2(\text{NO}_3)_2 \cdot 2\text{HCONMe}_2$	44.07	13.34	2.61	10.37
Obtained (avg 3 determinations)	44.02	13.29	2.66	10.35

The absorption spectrum of this compound from 2000 to 8000 Å differs markedly from that of uranyl nitrate hexahydrate and resembles that of anhydrous uranyl nitrate.⁽³⁾ This suggests that the orbitals involved in the binding of the DMF are different from those in the hexahydrate and that

the increased free energy of formation arises from this change in orbital type.

Synthesis of $\text{UO}_2(\text{CH}_3\text{COO})_2 \cdot \text{HCONMe}_2$ in a similar manner yielded bright yellow crystals decomposing above 160°C .

	Percent			
	U	C	H	N
Calculated for $\text{UO}_2(\text{CH}_3\text{COO})_2 \cdot \text{HCONMe}_2$	51.62	18.23	2.84	3.04
Obtained	51.67	18.20	2.79	3.06

Both compounds were very readily soluble in water and DMF and reacted with Li_2Pc in DMF to give an almost quantitative precipitate of UO_2Pc . Compounds of DMF with ThCl_4 and $\text{La}(\text{NO}_3)_3$ have also been observed, although not analyzed, and efforts to synthesize other DMF Lewis salts of lanthanide and actinide elements are in progress.

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PROGRESS REPORT: ROLE OF THE SMALL INTESTINE IN ACUTE RADIATION DEATH

I. Dose Effect on the Fourth Postirradiation Day

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

CF No. 1 female mice, 8 to 10 weeks of age, were exposed to single, whole-body doses of fission neutrons or Co^{60} γ -rays. Dose rates of approximately 5 rad/min of neutrons and 10 rad/min of γ -rays were used. Total doses throughout the lethal and supralethal ranges were given, with the highest dose at 3000 rad.

On the fourth postirradiation day, groups of mice at each dose level were sacrificed. Sections of intestines from the pyloric to the ileocaecal junctions were removed, and after flushing out the contents with saline, intestinal length was measured in saline solution under a standardized 14-g stretching force. Intestines were thoroughly dried for 24 hr at 100°C and weighed on a torsion balance.

With a few exceptions, 10 to 16 mice were used for each dose. Replication was necessary at higher doses in order to maintain this number of survivors on the 4th day. A total of 104 survivors are represented for neutron exposures and 139 for gamma-ray exposures. Unirradiated control mice numbered 348 animals; normal distributions were found for both intestinal weight and length in these animals, and the respective mean values were established as the control levels against which to assess the effects of irradiation.

The degree of damage to the intestinal mucosa as measured by loss of weight is shown in Figure 8, in which weights as percentages of control weights are plotted against dose in rads for each irradiation. At lower doses an initial "recovery" surge is seen after each of the two radiations. At doses above 350 rads of fission neutrons or 1450 rads of γ -rays no further recovery occurs. Weights drop precipitously to minimum levels at approximately 42% of control weight. Since little significant effect is seen with a further increase in dose, saturation of the effect is seen.

ED₅₀ (dose effective in 50% of the population) values in terms of the midpoint between control (100%) level and maximum effect (~42% occur at 71% of control weight). At this point the neutron ED₅₀ is 415 rads and that for γ -rays is 1795 rads. The RBE (n/γ) for intestinal weight loss on the 4th postirradiation day is thus 1795/415 or 4.33. Triangular points in Figure 8 represent the same neutron effect plotted in rem (rads x 4.33). Since the line formed by these points falls directly on the curve for the gamma effect, their slopes are equal, and the RBE of 4.33 holds over the entire dose range.

Figure 8. Intestinal weight response (% control values) to fission neutrons or Co⁶⁰ γ-rays on the fourth postirradiation day.

Changes in intestinal length on the fourth day following exposure to the two radiations are shown in Figure 9. A shortening of the gut becomes significant at doses above ~400 rads for neutrons and ~1600 rads for γ-rays. As the dose is increased above 1000 rads of neutrons the shortening effect becomes less pronounced. There appears to be no change at doses above the saturation level of 2000 rads of γ-rays.

Figure 9. Intestinal length response (% control values) to fission neutrons or Co⁶⁰ γ-rays on the fourth postirradiation day.

EFFECT OF NEUTRON DOSE RATE ON THE INCIDENCE OF MAMMARY TUMORS IN FEMALE SPRAGUE-DAWLEY RATS

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

Fifty-seven female Sprague-Dawley rats (approximately 60 days of age) were randomly divided into four dose-rate groups, and irradiated four times at weekly intervals with 100 rads of fission neutrons. The dose rates for the four groups are given in Table 11.

TABLE 11
Experimental plan

Group	No. of irradiated animals	Time of exposure, min	Position of boral shutter at CP-5 reactor	Dose rate, rads/min
A	14	85	25% open	1.2
B	14	34	50% open	3
C	14	17	100% open	6
D	15	3	Boral shutter pulled up out of thermal column	35

The purpose of this experiment was to determine whether the intensity of the fission neutron dose had effects on mammary tumor formation in the female rat. The rats were examined for tumors every two weeks and were weighed monthly. Tumors were measured with calipers for increase in size.

Results

Mammary tumors began to appear in each of the four irradiated groups within 2 to 3 months after the first neutron exposure. The cumulative frequency of mammary tumors is illustrated in Figure 10 for each of the four experimental groups for the 200-day period following the first exposure; 40% of the neutron-irradiated rats developed mammary tumors during this period.

Figure 10
Mammary tumor incidence in female Sprague-Dawley rats given 4 weekly exposures to 100 rads of fission neutrons at different dose rates

Although these results must still be considered preliminary, there appears to be a neutron dose-rate effect on mammary tumor formation in the female rat. At the time of this report, only 3 of 15 rats exposed to the neutron dose at the high rate of 35 rads/min have shown any visible mammary tumors; no new tumors appeared in this group (D) in the period between 100 and 200 days after exposure. In contrast, 8 of the 14 animals of Group A, exposed at the low dose rate of approximately 1 rad/min, have shown tumors. The incidence of new tumors in this group appears to be still increasing.

No mammary tumors have yet appeared (at 200 days) in the 35 non-irradiated control rats. Although most of the tumors have grown in size throughout the period of observation, four regressed and eventually disappeared. Four of the irradiated animals developed two tumors. At the time of this report, 90% of the neutron-irradiated rats are alive; 6 rats died with large ulcerating tumors in the period between 150 and 200 days after the first neutron exposure. Most of these neutron-irradiated rats already show radiation cataracts.

Comparable observations are being made in a similar group of female rats irradiated with Co^{60} γ -rays. In this case, the animals were exposed to 200 rads weekly for four weeks at four dose rates similar to those used in the neutron exposures. A detailed comparison will be made.

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

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

Five groups of CF No. 1 female mice, 2 to 3 months old, were irradiated at the CP-5 reactor with single, whole-body doses of fission neutrons. The mice received total doses of 20, 50, 75, 100, or 150 rads delivered at a dose rate of approximately 5 rads/min.

Within one week after exposure the irradiated female mice were mated with unirradiated males (4 female mice caged with each male). Pregnant mice were isolated and remained with their young until weaning, approximately a month after birth. Then the irradiated females were returned to the males for a second mating test. This procedure has been repeated so that the mice are now on their fourth breeding test, approximately 6 months after the neutron exposures.

The results of the first three fertility tests are summarized in Table 12. It is evident from these data that a single irradiation of 50 rads of fission neutrons causes a significant and progressive decrease both in the percentage of pregnancies and in average litter size. Exposure of 75 rads or more soon produces complete sterility. On the other hand, the lowest dose utilized, 20 rads of fission neutrons, does not appear to produce significant effects either on the percentage of pregnancies or on the average size of the litters produced during the first three mating tests.

The young that were successfully weaned are being retained in the laboratory in order to study the effects on longevity of the progeny of irradiated female mice exposed to varying doses of fission neutrons.

TABLE 12

Neutron effects on fertility in CF No. 1 female mice

Radiation dose, rads	Test No.	No. female mice mated	Pregnancies		Average litter size
			No.	%	
Control	1	20	19	95	10.5
	2	20	20	100	12.2
	3	20	20	100	10.2
20	1	15	15	100	10.4
	2	15	15	100	10.6
	3	15	13	86	10.9
50	1	16	14	87.5	7.6
	2	16	12	75	3.7
	3	16	3	19	2.7
75	1	16	14	87.5	7.6
	2	16	3	19	3.3
	3	16	0	0	0
100	1	11	11	100	6.4
	2	11	0	0	0
	3	11	0	0	0
150	1	16	7	44	3.6
	2	16	0	0	0
	3	16	0	0	0

MEASUREMENT OF Cs^{137} IN TEA AND HUMAN MILK

S. S. Brar, P. F. Gustafson, and M. A. Kerrigan

Many foods and beverages including tea have been surveyed for radioactivity by the Food and Drug Administration, using gross β -counting methods. Since tea has somewhat higher radioactivity than some of the comparable beverages, a random sample of bulk tea has been surveyed for its Cs^{137} and K^{40} content by γ -ray spectrometry.

Bulk tea procured through the cafeteria of the Laboratory was divided, for study purposes, into three equal portions. The activities of these portions agreed within $\pm 1\%$. Each portion of tea was then given a different treatment, dried, and remeasured. Portion 1 was washed thoroughly in cold water; portion 2 was washed in cold water to which a strong liquid detergent had been added; portion 3 was washed in hot tap water (approximately 140°F). Table 13 shows the results for various measurements.

TABLE 13

 Cs^{137} and potassium content of bulk tea

Portion	Weight of sample measured, g	Cs^{137} in untreated tea, $\mu\mu\text{c}/\text{kg}$	Cs^{137} in tea after treatment, $\mu\mu\text{c}/\text{kg}$	Potassium in tea before treatment, g/kg	Potassium in tea after treatment, g/kg
1	363	1558	1259	0.76	0.39
2	363	1577	944	0.75	0.30
3	363	1572	855	0.77	0.12

From these results it is estimated that not more than 2 $\mu\mu\text{c}/\text{cup}$ (four ounces of liquid) is consumed from this sample of tea if the tea is made according to the above specifications, while in 1959, the Cs^{137} concentration in the Chicago area milk varied from 4 to 13 $\mu\mu\text{c}/\text{four ounces of milk}$.⁽¹⁾

One sample of Darjeeling tea was also measured and found to contain about one-third as much as the bulk tea.

While untreated bulk tea shows approximately 2100 $\mu\mu\text{c}/\text{g}$ of potassium, the tea made with hot water shows only 1100 $\mu\mu\text{c}/\text{g}$ of potassium.

A 1-liter sample of human milk (procured from Northwestern University through the assistance of Dr. C. F. Decker of this division) was measured for its Cs^{137} content, which was compared with that of cows' milk available in the cafeteria. The human milk was found to contain only one-seventh as much Cs^{137} as the cows' milk.

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LATENCY AND GROWTH OF OSTEOSARCOMAS INDUCED BY RADIOSTRONTIUM

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

The latent period is defined as "the period elapsing between the application of a stimulus and the obvious response."⁽¹⁾ For some time we have been concerned with the latent period for the induction of osteosarcomas by radioelements that deposit in bone. The true interval should lie between the time when the tissue has absorbed the required amount of energy and the time when that tissue has undergone neoplastic transformation. The present experiment relates primarily to the end of the latent period, not to its beginning.

Our previous studies of the carcinogenic response of mice to bone-seeking radioisotopes have relied upon roentgenographic detection of skeletal lesions at the time of death and their subsequent histopathologic examination. The shortest observed interval between administration and death with a tumor has decreased as the number of animals that have been examined has increased. At present it stands at 154 days. Since a tumor present at death must have originated some time before death, it is obvious that 154 days after injection cannot represent the actual end of the latent period. It seemed that periodic roentgenographic examination of living animals would make it possible to establish the shortest interval between injection and roentgenographic evidence of malignancy. At the same time this technique would provide information on tumor growth that might be useful in estimating the time when the neoplastic change occurred.

Materials and Methods

Our standard test system for assaying radioisotope toxicity has been a single intravenous injection into 70-day-old CF No. 1 female mice. Under these conditions, $1.0 \mu\text{c Sr}^{90}/\text{g}$, given in equilibrium with Y^{90} , produces an average of more than four osteogenic sarcomas per mouse. This procedure and injected dose were used in the present study, which involved 29 CF No. 1 females and 24 CBA females. The former strain received an average of $0.9 \mu\text{c/g}$ (0.81 to 1.03) and the latter an average of $1.1 \mu\text{c/g}$ (0.96 to 1.23).

X-ray pictures were taken 1, 2, and 3 months after injection and at weekly intervals thereafter. The final picture was taken at autopsy after all the viscera had been removed. By retrogressive comparison of the roentgenograms, the sarcomas present at death could be traced back to their first appearance. Whether a specific lesion was neoplastic or not often was difficult to determine, and an arbitrary decision had to be made.

If the cortex was incomplete, malignancy was diagnosed. If the cortex was not broken, but the general appearance suggested very early malignancy, the lesion was classified as a questionable tumor. Diagnoses of such areas present at death will eventually be based on histopathologic examination, and the information gained in this fashion should make it possible to classify more accurately many of the lesions detected before death.

Studies of tumor growth were based on estimates of tumor volume derived from planimeter measurements of successive X-ray pictures. Tumors with areas of 0.4 cm^2 or more were measured quite accurately, but more precise methods will have to be devised for the smaller lesions. Consequently, any tumor that did not reach 0.4 cm^2 , or 0.19 cm^3 , could not be included in the present analysis of tumor growth.

Results

The type of material with which we were working is illustrated in Figure 11. At 180 days after injection there was a dense area in the right iliac crest and the outline of the left lateral process of the second sacral vertebra was slightly irregular. These were classified as questionable tumors. By 208 days they were frank sarcomas, and their subsequent growth was rapid. The progressive changes in the distal femurs were classified as questionable sarcomas from 201 days to death, 241 days after injection.

The first sarcoma was diagnosed in a CF No. 1 mouse 86 days after injection. The number increased rapidly, and by 252 days there was an average of 4.6 tumors per mouse. The cumulative incidences of tumors in the two strains are shown in Figure 12. The solid lines represent the tumors as they appeared roentgenographically; the dotted lines represent tumor incidences as they are usually presented, i.e., at the time of death of the animal. The distance between the solid and dotted lines for each strain is about 30 days on the abscissa and between 1.5 and 2 tumors on the ordinate. Although the slopes of the incidence curves and the total number of tumors per mouse are the same for the CF No. 1 and the CBA mice, the values for the latter are displaced about 50 days to the right.

Tumor rates also were very similar in the two strains, but they showed the same 50-day displacement. The values plotted in Figure 13 are the daily averages over 28-day periods, recalculated every 7 days. These rates, based on the time of roentgenographic appearance of tumors, have one feature that is markedly different from the usual method of representation based upon tumors at the time of death. In the latter case the rates ordinarily continue to increase until the population has been reduced to one or two individuals.

Figure 11. Roentgenograms of a mouse taken 180, 208, 221, and 236 days after injection of Sr^{90} . Note development of lesions of right iliac crest, second sacral vertebra, and distal areas of femurs.

Figure 12. Cumulative incidence of osteosarcomas as detected on X-ray pictures (solid line) or counted at the time of death (dashed line).

Figure 13. Rate of roentgenographic detection of osteosarcomas.

In the present instance they declined almost as sharply as they had originally increased after maximum values had been reached (196 days for the CF No. 1 and 244 days for the CBA mice). The decline occurred in spite of the fact that about 15% of the injected radiostrontium still remained as a source of radiation in the skeleton. Thus, an increase in the total energy absorbed was not associated with an increased appearance of tumors. It would seem that the required stimulus had been applied some time before the first tumor was recognizable on X-ray and that tumor appearance thereafter followed a normal frequency distribution pattern with time. Once set into motion, the process was not influenced by the continuing radiation. Perhaps at this level of exposure the carcinogenic stimulus reached saturation relatively soon after injection, and subsequent radiation was not capable of increasing the carcinogenic response. Or, perhaps additional stimulation was possible, but the continuing radiation was delivered at an ineffective dose rate.

The locations of the sarcomas and the times of their appearance were examined for clues to the underlying difference between the two strains. The same 50-day shift to the right for the CBA's is noted in Figure 14, but the location of the tumors in each strain does not seem to be associated with time after injection. For example, tumors in the femur or spine occurred both early and late in each strain, and there was no tendency for certain locations to present neoplasms more often at one interval than at another.

Figure 14. Frequency distribution of osteosarcomas and their locations as detected on X-ray examination.

However, more tumors arose in some bones than in others. The 29 CF No.1 mice had 134 positive tumors, and the 24 CBA's had 107. When the population sizes were normalized at 29 for the comparisons illustrated in Figure 15, the CBA tumor number was raised to 129. The odds against the strain differences in the number of tumors in the tibia and in the lumbar spine being due to chance are 143 to 1 and 46 to 1, respectively. None of the other strain differences are statistically significant. The mechanism responsible for this variation between the strains in tumor location is not known, but it deserves investigation since it might also be responsible for the variation in carcinogenic response of different skeletal sites within the same strain. For example, many more tumors are consistently located in the femurs of mice than in the humeri in spite of the fact that the energy absorbed by these two bones is the same.

Figure 15. Location of bone malignancies in the two strains of mice. The locations as listed from left to right are: femur, tibia, humerus, cervical spine, thoracic spine, lumbar spine, sacral spine, caudal vertebrae, pelvis, skull, and rib.

Figure 16 compares the length of time the lesions existed in each strain before the animals died. The average interval, beginning with the time a lesion was first diagnosed as possibly neoplastic, was 38 days among the CF No. 1 mice and 44 days among the CBA's. This difference of six days was reduced to 3.6 days when the durations of only the questionable lesions was compared, and to 1.3 days when only the positive sarcomas were compared. These are small variations, but they suggest that tumor development may have progressed somewhat more slowly among the CBA mice.

Figure 16. Average time between diagnosis of malignancy and death. + = positive osteosarcomas; ? = possible or probable osteosarcomas.

The measurements of tumors on successive weeks demonstrated that the rate of growth of individual tumors was exceedingly variable. Several generalizations can be made: (1) Tumors that appeared early in the experiment were just as likely to grow slowly, or to grow rapidly, as were tumors that made their first appearance late in the experiment. (2) Tumors that grew very slowly for a long period of time were just as likely to continue to grow slowly as they were suddenly to start to grow rapidly. (3) Once a tumor had begun to grow rapidly, it continued to do so. (4) There was no apparent association between rate of growth and location.

The proportions of tumors that attained various volumes are shown in Figure 17. Sixty-three percent of the CBA tumors and 57% of the CF No. 1 tumors did not reach measurable size (0.19 cm^3) with the present technique although some of these could be followed as definite malignancies for 7 or 8 weeks. Contrarily, other tumors grew to 10 cm^3 or more during the same interval. The proportions of measurable tumors that reached a given volume by the time of death was quite similar in the two strains.

The tremendous variability in time of appearance and in growth rate among the individual tumors complicated comparisons between the two strains. One approach was to eliminate the time variable by starting with the week of first positive diagnosis, and to examine the growth of only those sarcomas that reached 0.19 cm^3 by the time the host died. There were 46 CF No. 1 and 29 CBA tumor masses available for this study. In some instances tumors had intermingled so completely that the combined mass had to be measured. The average tumor volumes plotted against weeks from first positive diagnosis are presented in Figure 18. None of the CBA tumors had reached measurable size by the time they were first frankly neoplastic, and at successive weeks their average size remained less than that of the CF No. 1 tumors.

Figure 17. Grouping of tumors according to size at the time of death. The proportion of tumors in each group exceeding 0.19 cm^3 is based upon the total number of measurable tumors.

Figure 18. Average tumor growth rate as a function of time after roentgenographic diagnosis.

The open circles in the graph indicate where the CBA values would fall if their time scale were reduced by one week. Perhaps a more logical correction would have been six days rather than seven since the average time between first appearance of a possible tumor and death was six days longer in the CBA strain than in the CF No. 1 strain. A six-day reduction of time places the CBA values even closer to the CF No. 1 line, and there seems to be no difference in the growth rate of CBA and CF No. 1 tumors after they have reached 0.19 cm^3 . The combined values fall on a line described by $y = ax^{3.04}$. When the tumors smaller than 0.19 cm^3 have been measured and have been added to these data, the values of the first few weeks will be larger, and the slope of the line in this area will decrease. With proper information at this end of the growth curve, it may be possible to estimate both the actual time when neoplastic growth began and the size of the original neoplastic mass.

In a continuation of our attempt to define the difference between the two strains, we examined the total volume of neoplastic tissue growing within each population as a function of time after injection. These values are plotted in Figure 19. Data will be added in the direction of zero time when the smaller tumors have been measured. The present data show a very striking difference in the rates of increase of the total masses of neoplastic tissue within the two populations. Since the average rate of growth of the sarcomas in each strain was the same after they had reached 0.19 cm^3 , the difference must be associated with growth before this size had

Figure 19. Growth rate of total neoplastic mass within the population as a function of time after injection.

been attained. These results cannot be attributed to differences in the moment marking the end of the latent period. If this were a latent period effect, the two curves would have different origins but similar slopes. On the contrary, they display very dissimilar slopes, and it is possible that they have a common origin.

The combination of present results in this experiment leads to the impression that the sarcomas in the CBA and the CF No. 1 mice were identical, but those located in the former were confronted with a more adverse environment, which retarded their early development. However, once they attained a certain size, the resistance of the environment was overcome, the host influence was lost, and their subsequent growth followed the same pattern as that displayed by the CF No. 1 tumors.

Summary

1. CF No. 1 and CBA mice responded to comparable amounts of injected radiostrontium with comparable numbers of osteogenic sarcomas. However, tumor appearance in the latter strain lagged behind the former by about 50 days.
2. The rate of tumor appearance, determined by X-ray examination of living mice, declined sharply after a maximum had been reached in spite of the fact that radiation was continuing.
3. The location of the tumors was not associated with time after injection. The tibias and the lumbar spines of the two strains responded with different numbers of tumors.
4. The rate of growth of individual tumors was exceedingly variable. It was not associated with time after injection or with time after roentgenographic appearance. However, the average rate of growth after positive diagnosis followed a power function, which was the same for both strains.

5. The total volume of neoplastic tissue increased three times as rapidly in the CF No. 1 population as in the CBA population. It is suggested that this represents a difference in rate of growth and development imposed by the host environment during the time marked by the end of the true latent period and the attainment of a certain minimum size.

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RADIUM DAMAGE IN MOUSE BONE

Miriam P. Finkel and Birute O. Biskis

The histologic material collected during the study of the toxicity of Ra^{226} in mice is currently being examined. The present report is concerned with the sequence of pathologic change in the skeleton. The osteogenic sarcomas have been discussed elsewhere,⁽¹⁻⁵⁾ and they will be considered here only as the description of the general picture of bone damage requires.

The radium was given in isotonic chloride solution at pH 2 to 4 in a single intravenous injection to CF No. 1 female mice that were approximately 70 days old. The amount of material administered and the number of animals are listed in Table 14. The mice were sacrificed when moribund, and their skeletons were preserved in formaldehyde. Besides routine sectioning of the right femur and the thoracic and lumbar spines, all areas with positive or possible pathology, as detected on roentgenograms taken before preservation, were selected for histological examination. Embedding in celloidin and staining with hematoxylin, eosin, and azure were the usual procedures.

TABLE 14

Experimental plan and some pertinent tumor data

Amount injected, $\mu\text{c}/\text{kg}$	Number of animals	Time of first tumor death, days	Percent of surviving population with tumors
4170	13		
2080	15		
1040	30		
762	30		
521	30		
288	45		
100	45	317	86
50	45	287	50
28.8	60	232	46
12.0	75	289	37
6.0	90	356	31
3.0	90	379	17
1.2	105	303	14
0.6	120	476	2.4
0.0	210	528	1.9

Description of Pathologic Change

The following summary of the histopathologic changes in the skeleton uses the femur as the model. Specific mention is made of the vertebrae only when markedly different processes were noted there. The mice were not sacrificed on any prearranged schedule. Nevertheless, a rather good time series of autopsy material is available for study due to the fact that, while the experiment was still quite young, the animals suffered from an endemic intestinal infection that rather sharply increased the early mortality rate.

4170 μ c/kg (8 to 13 days). Most of the lacunae in the trabeculae and in the metaphyseal cortex were empty. A few spindle cells were present in the metaphyseal marrow.

2080 μ c/kg (12 to 24 days). Empty lacunae appeared in the trabeculae and in the metaphysis, and by 24 days even in the diaphysis. Spindle cells within the marrow cavity were associated first with coarse pink fibers and later with basophilic intercellular material. Small foci of atypical fibrous bone appeared in the metaphysis.

1040 μ c/kg (6 to 64 days). Empty lacunae appeared only in the trabeculae up to 30 days, but by 40 days the diaphysis was involved also. No osteocytes remained after 60 days. Spindle cells were present in the metaphyseal marrow at 15 days and in the diaphyseal marrow as well by 28 days. They were followed by pink-staining fibers and the formation of fairly regular bone, which only later became attached to the endosteum. By 64 days the new bone extended to the mid-shaft.

Atypical fibrous bone, consisting of pink fibers, basophilic fibers, and basophilic intercellular substance, was present in the metaphysis in varying amounts from 12 to 24 days. The smallest foci were free in the relatively empty marrow cavity. As they enlarged they joined the old trabeculae, and between 27 and 64 days they fused to form a wide band across the metaphysis. The epiphyseal border of this band extended as far as the epiphyseal cartilage, and it was more fibrous and basophilic than the diaphyseal border, which became very dense and completely sealed off the metaphysis after 60 days.

In a few animals the cartilage was very hyperplastic, but the columnar arrangement of the cells remained uninterrupted.

762 μ c/kg (14 to 97 days). The sequence of events was much the same as that noted after 1040 μ c/kg. However, after 80 days the band of atypical fibrous bone across the metaphysis was dead, and only a few osteocytes were seen near its diaphyseal border.

521 $\mu\text{c}/\text{kg}$ (8 to 164 days). Empty lacunae appeared only in the trabeculae and metaphyseal cortex up to 50 days. By 70 days the diaphyseal cortex was also involved, and by 100 days few, if any, osteocytes remained.

Cellular new bone appeared in the diaphyseal region of the metaphysis by 15 days, and by 50 to 70 days it was present along the endosteum of the entire shaft. After 100 days, however, this new bone showed signs of death, beginning primarily in the epiphyseal border of the metaphysis. The diaphyseal border, which contained a few osteocytes, remained more solid, and it appeared to seal the metaphysis from the diaphysis.

Atypical fibrous bone in the metaphysis was associated with the old trabeculae, which contained remnants of calcified cartilage and which were outlined with basophilic fibrous material.

288 $\mu\text{c}/\text{kg}$ (9 to 231 days). The progression of osteocyte death in the original bone was similar to that noted after 521 $\mu\text{c}/\text{kg}$. Between 15 and 100 days new cellular bone appeared between the dead trabeculae, which contained remnants of calcified cartilage and which were outlined by basophilic fibrous material. This new bone died, more new bone was laid down, which also died, and this process gradually filled the spaces between the original trabeculae so that a solid bar composed of old and new dead bone extended across the metaphysis. A few osteocytes remained in the diaphyseal border of this bar at about 125 days. The diaphyseal marrow in the region remained acellular as if cells were prevented from coming too close to the metaphysis. Later the metaphyseal bar seemed to be "polished" on its diaphyseal border, and by 190 days a slightly concave, horizontal bony plate was present in this area. While this process was going on at the distal end of the femur, small amounts of cellular new bone could always be found in the middle of the diaphysis.

By 200 days the new bone was dead and a heavy blue line surrounded many regions. Only a few osteocytes were present in the endosteum, and there was very little endosteal bone. Resorption of the metaphyseal bar, which seemed to start as a small focus in the central portion of the cartilage, began after 200 days. In some cases the bar was completely gone by 200 to 230 days, and the bone marrow extended as far as the epiphyseal cartilage. In spite of this rapid resorption, no osteoclasts or characteristic lacunae were seen.

The cartilage was very proliferative as early as 21 days, and resorption was already apparent. The cartilage next to the epiphysis was very narrow and irregular, but the columns of cells were tall and regular. The first evidence of destruction appeared in these columns. A small group of cells would die and degenerate, leaving amorphous, blue-staining debris. As the debris was removed, empty spaces containing a few spindle cells and

erythrocytes took its place. By 125 days the larger spaces contained blood vessels, clumps of bone marrow, and spicules of new bone. In the final stage of this process, which was seen in varying degrees from 15 to 230 days, and which also included the removal of the metaphyseal bar described above, a thin bony plate lined the remaining narrow band of irregular cartilage.

100 μ c/kg (82 to 370 days). Empty lacunae were found only in the trabeculae up to 90 days and in the metaphysis and adjacent diaphysis as well at 100 days. Many osteocytes were still present up to 300 days, but after that time, the bone appeared to be quite dead.

At 80 days the metaphysis contained a bar of dead new bone joining the old trabeculae, which held remnants of calcified cartilage. This bar was located adjacent to the cartilage, whereas after higher levels of exposure it had formed some distance from the cartilage. Resorption of the bar started as early as 82 days, and remnants were still being removed as late as 360 days. There was considerable variation among the individual animals. The remaining metaphyseal cortex showed remnants of atypical fibrous bone and of calcified cartilage. Osteoclasts and characteristic lacunae were absent.

The cartilage at 80 days was narrow and irregular, and it was enclosed by a horizontal bony plate. Severance and resorption were seen as early as 80 days and as late as 350 days. In general, after 200 days the epiphyseal cartilage had been resorbed and the bone marrow extended into the epiphysis.

New cellular bone was seen at all times but in small amounts only, primarily in the lumbar vertebrae and femoral epiphysis. Very little new bone appeared in the shaft of the femur. In general this new bone had a normal, pink-staining matrix and regular, small cells.

Six of the seven animals that died after 317 days had osteogenic sarcomas.

50 μ c/kg (42 to 445 days). Empty lacunae were numerous in the metaphysis between 70 and 100 days, and by 174 days a few osteocytes appeared only in a small area adjacent to the cartilage and in the diaphyseal cortex. Even fewer were present after 244 days.

Portions of a metaphyseal bar were seen between 70 and 244 days. The amount of new bone varied from animal to animal, seemingly unrelated to time. The vertebrae and femoral epiphysis showed relatively more new bone, and the femoral shaft relatively little. After 400 days the bone was rather sclerotic, the metaphyseal bar was gone, and osteocytes were present only in the proximal femur.

Fourteen of the 28 animals that died at 287 days or later had osteogenic sarcomas.

28.8 $\mu\text{C}/\text{kg}$ (54 to 577 days). A few empty lacunae appeared in the femoral metaphysis and in the vertebrae between 50 and 100 days. They had increased by 200 days, and after 300 days they were also present in the diaphysis. The proximal portion of the femoral cortex remained more or less regular at all times. A small area of new bone appeared in the metaphyseal cortex just below the cartilage, and the dead bone seemed to be moved away. No atypical fibrous bone or metaphyseal bar were seen at any time.

Osteogenic sarcomas appeared in 25 of the 54 mice that died at 232 days or later. Most of the tumors were too large at autopsy for guessing their site of origin, but a few seemed to begin in the new endosteal bone in the distal metaphysis of the femur. No proliferative activity was seen in corresponding or in adjacent areas.

12 $\mu\text{C}/\text{kg}$ (88 to 660 days). After 100 days a few empty lacunae were seen in the metaphysis. By 200 days the area adjacent to the cartilage was still cellular, but by 300 days many osteocytes in the diaphyseal border of the metaphysis and in the vertebrae had died. By 400 days a few holes were seen in the diaphysis as well.

The epiphyseal cartilage closed between 80 and 200 days. In some cases resorption occurred between 300 and 400 days, and this was followed by the formation of active new bone, especially in the vertebrae.

Twenty-five of the 67 mice that died at 289 days or later had osteogenic sarcomas.

6 $\mu\text{C}/\text{kg}$ (5 to 800 days). The epiphyseal cartilage closed between 111 and 136 days. Empty lacunae appeared first between 200 and 300 days in the diaphyseal border of the metaphysis and in the vertebrae. Their number increased slightly between 300 and 400 days, though the picture was not consistent. Between 300 and 500 days the bone varied from regular to partially dead, but after 700 days there was an abrupt change to quite dead bone and cartilage. Over 50% of the lacunae were empty, especially in the epiphysis and metaphyseal border of the diaphysis of the femur and in the vertebrae. In general the cartilage had been replaced by bone.

Although very little proliferation was seen, sixteen of the 51 mice that died between 356 and 800 days had osteogenic sarcomas.

3 $\mu\text{C}/\text{kg}$ (73 to 692 days). The bone was regular; the cartilage was closed and replaced by bone. After 300 days there were a few empty lacunae, primarily in the metaphyseal border of the diaphysis. More appeared throughout the bone after 600 days, but this may have been the result of age rather than of radiation.

The first death with a tumor occurred at 379 days. There were a total of 9 deaths with bone malignancies among the 52 mice that lived to that time.

1.2 μ c/kg (6 to 815 days). The bone was regular, and no deviations from normal were noted. Empty lacunae were a constant finding after 700 days. Many animals displayed active bone growth after 500 days, particularly in the vertebrae, where it often coincided with the resorption of cartilage, and in the proximal end of the femur.

Of the 66 mice that died between 303 and 815 days, 9 had osteogenic sarcomas and 3 had skeletal lesions that may have been neoplastic.

0.6 μ c/kg (10 to 766 days). The bone appeared to be similar to that of the untreated animals in all respects. There was one bone tumor at 476 days, when the population consisted of 41 animals, and a possible malignancy at 560 days. This proportion falls within the normal range for control CF No. 1 female mice.

Discussion

The preceding descriptive narrative can be summarized as follows:

1. Osteocytes may be killed by radium very rapidly, or, when the amount administered is reduced manyfold, they may die only after a relatively long period of time.
2. Bone responds to acute radium damage by forming new bone, not from the endosteum but within the marrow cavity. Spindle cells are followed by coarse fibers, which are in turn followed by more or less regular bone, or, alternatively, foci of atypical fibrous bone appear between the trabeculae and eventually fuse with them. The new bone is not immune to radiation damage, and it, in turn, may die.
3. Bone repair progresses slowly, and one is impressed with the lack of proliferative activity such as follows damage from plutonium and radiostrontium. Either radium damage does not elicit the same response as damage from other bone-seekers, or the same response cannot be achieved because the cells that should bring it about have been destroyed or are destroyed as they enter the damaged area.
4. Damage to the cartilage is less severe, and it appears to be indirect. The cells continue to proliferate, but since transformation to bone does not occur, long columns result. Eventually these break down and are resorbed.

5. The first death with an osteogenic sarcoma occurred 232 days after a moderate amount of radium had been administered. At higher levels of exposure the first tumor deaths occurred later. The osteogenic sarcomas were not associated with the hyperactivity in adjacent bone that is commonly observed after the administration of Sr^{90} , Ca^{45} , and Pu^{239} .

The radium tumor data have always been something of an enigma because they are strikingly different from those that appear after the administration of other bone-seekers. With radium the tumor deaths occur later, the total number of tumors is much smaller, and the dose-response curve is unique in that it is a plateau with both sides rapidly decreasing to zero. The clue to these contrary results may lie in the tremendous damage produced by radium, which appears to be much more severe and perhaps even of a different nature from the damage produced by the other bone-seekers. That radium is so much more toxic is not surprising in view of the results of the dose rate measurements that have been made of Ra^{226} , Sr^{90} , and Ca^{45} in mouse bones at the microscopic level.⁽⁶⁾

The radium data may be explained in the following manner. First, let us assume that the number of osteogenic sarcomas increases with increasing amounts of injected radium, that the latent period is constant, and that the appearance of tumors occurs with a normal distribution pattern with time. Let us further assume that the neoplastic process is independent of the damage we have described, which occurs almost immediately after injection, which is less severe with smaller amounts of injected radium, and which is repaired more quickly when the initial injury is less severe. Then, by a proper balance of these two processes of carcinogenesis and damage, it can be demonstrated that at certain levels of exposure the time to death with a tumor should increase with increasing amounts of radium, that the number of tumors that can appear is limited, and that the incidence will, in fact, decrease with increasing level of exposure. This hypothesis is represented graphically in Figure 20.

There is no difficulty in distinguishing damage that is lethal to the cell from change leading to malignancy since a dead cell cannot become a neoplastic cell. However, there is no such sharp distinction for cellular injury that is less than lethal. Nevertheless, it is possible that damage in the usual sense is not the precursor of the neoplastic change and that the two events are independent. In this radium experiment, as in many other studies with bone-seeking isotopes, maximum tumor incidence did not occur among the animals showing maximum damage. On the contrary, tumor incidence was high when there was little other evidence of radiation damage.

Figure 20

Hypothetical representation of interaction of two independent processes, injury in the usual sense and carcinogenesis.

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PROGRESS REPORT: DYNAMICS OF CELLULAR POPULATIONS

G. A. Sacher and E. Trucco

Previously, preliminary steps were given for the development of mathematical models for the regulation of blood cell populations.⁽¹⁾ Plans were also discussed for further investigating properties of the model (Equations 8 in op. cit.) for several different sets of values of the unknown parameters n , λ , and μ . This study has been completed and is reported here. Equations 8 were solved on the PACE Analog Computer; we took $n = 10$; the remaining two parameters; λ and μ , were assigned, independently, each of the following values:

$$\begin{aligned}\lambda &= 2, \text{ or } 10, \text{ or } 50; \\ \mu &= 1.0864, \text{ or } 2, \text{ or } 10;\end{aligned}\tag{1}$$

so that altogether we have examined nine main cases. The value of β_0 [i.e. β for $I = 0$ (see Reference 1, top of p. 51),] was chosen to be 3.0 since considerations on the turnover time of cells in the proliferative compartment make it seem likely that β_0 should be at least that large. In each of the above cases we then varied β (usually in steps of 0.2) between $\beta = 0$ and $\beta = \beta_0 = 3.0$.

As mentioned in Reference 1, the solution recorded by the computer did quite frequently show instability in the form of limit cycles around the steady state values. This happened, broadly speaking, whenever the coupling between the proliferative and the final compartment was relatively large (i.e. when λ and/or μ were sufficiently small). Altogether we found complete absence of limit cycle instability in the whole range of β ($0 \leq \beta \leq 3.0$) only if the two inequalities $\lambda \geq 10$ and $\mu \geq 2$ were satisfied simultaneously. This means that this particular system is inadequate to describe the behavior of a cellular population for realistic values of the parameters. One essential feature of this model, as already stated, (Reference 1, p. 48) is that it has only one feedback loop, acting from the peripheral upon the proliferative compartment. Our results tend to show that such a feedback loop is not sufficient to insure dynamic stability.

A second important qualitative property of the model was the distribution of maturation times around their mean, τ . In Equations 8 of the model,⁽¹⁾ the third equation ($i = 2, 3, \dots, n-1$) represents, formally, a time-delay chain. The over-all average time delay τ is given by $\tau = (n-1)/c$. If n , i.e. the number of equations, is changed, the mean time lag will remain invariant. This is because, in evaluating the coefficients, we have chosen our parameter c proportional to $n-1$, so as to leave the steady-state throughput unchanged (cf. Equation 23, Reference 1). However, as we have already noticed (Reference 1, p. 49) the distribution of maturation times around the

mean, τ , will vary with n , being exponential when the delay chain consists of only one element, and approaching a delta function if $n \rightarrow \infty$. In the following we shall refer to it as the time-delay distribution. The case $n = 2$ represents a purely random emergence of cells into the circulation, while $n \rightarrow \infty$ corresponds to a "first in-first out" situation.

In a follow-up study we have, therefore, examined the same model, but with $n = 2$ instead of $n = 10$. All the other parameters were given the same values as in the previous case, the only change thus being in the time-delay distribution. It turns out that, while this special case is certainly less realistic than the first one (for we know from tracer studies that the over-all time delay distribution between the proliferative and the peripheral compartment is not exponential), its stability properties are much improved. Here we found instability in the range $0.7 < \beta < 2.1$ only if $\lambda = 2$ and $\mu = 1.0864$, but complete absence of limit cycles (when β varied from zero to β_0) for all the other eight combinations of parameter values specified previously.⁽¹⁾

We have also examined a third model, whose feedback characteristics differ from those of our previous systems in two respects:

- a) The number of maturing cells limits the rate of production in the proliferative compartment.
- b) A decrease of N_n (circulating cells) directly facilitates migration into the terminal compartment.

The total number of compartments was taken equal to three; this model, then, is also unrealistic with respect to its time-delay distribution. However, it is difficult, with our present equipment, to set up the n -step system of this type with a value of n large enough to approximate the real time-lag distribution. In our future work the computational program will be modified so as to circumvent this difficulty.

In this third case we used a digital rather than an analog computer program. The original equations were linearized by means of a Taylor expansion in the neighborhood of the steady states, and the stability properties were then investigated in the well-known manner by calculating the roots (s_1 , s_2 , and s_3 , say) of the "characteristic equation." In contrast to the other two models, this last system turned out to be quite stable over a wide range of parameter values, thus demonstrating the importance of appropriate feedback connections for steady-state stability. However, these results are by no means complete; we cannot yet state with confidence which particular type of feedback is either actually realized in nature or most desirable from a theoretical point of view. Much more research work (both theoretical and experimental) is still needed on this subject.

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MECHANISMS OF RESISTANCE AND REVERSAL IN THE INITIAL RADIATION RESPONSE OF THE CHICK

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

Many aspects of restitution are embodied in the concept of "biological recovery" from the effects of ionizing radiations. Responses observed under various exposure conditions (protracted, split, or fractionated doses) indicate that two general classes of radiation-induced processes actively influence this "recovery" phenomenon: (1) reversal processes that reduce the radiation effectiveness and (2) resistive processes that increase the tolerance of radiosensitive systems. In the γ -irradiated chicken, analysis of the initial mode of death, which appears within two days after exposure, emphasizes the dependence of mortality on over-all time of irradiation. From studies of dose-time relations for mortality following a series of protracted Co^{60} γ -ray exposures ranging from a few minutes to 24 hours, a zero-order reversal process has been postulated to operate in the 3-day chick.⁽¹⁾ When the over-all time of exposure is varied by means of a split-dose technique, using high-rate exposures and intervenient periods up to 1440 minutes, mortality in the initial period indicates that an increase in resistance to radiation injury develops between exposures. The resistance reaches a maximum after an interval of about 300 minutes and decreases after longer intervenient periods. At the time of maximum resistance following an initial dose of 700 r Co^{60} γ -rays, increase in radioresistance, in terms of dose, is equal to more than 2000 r. Loss of resistance is rapid between 360 and 960 minutes, but a residual amount after 1440 minutes is approximately equal 300 to 400 r.

The course of the loss and subsequent increase in radiosensitivity after an initial dose of 700 r is quantitatively described by the relationship between the interval separating the exposures and the dose required for 50% mortality (Figure 21).

Figure 21

Dose required for 50% mortality at intervenient periods through 1440 min after an initial exposure to 700 r, Co^{60} γ -rays, as interpolated or extrapolated from probit mortality curves.

The kinetics of the resistive process through 300 minutes is complicated by the possible action of reversal processes that may operate after exposure. If a lag in the initiation of the resistance is postulated, as is suggested from results of several experiments involving a protracted exposure immediately followed by a high-rate challenging dose, then it may be inferred that reversal processes continue after an exposure at the same rate as during irradiation. Additional information is needed in order to determine the course of the loss of resistance. The deceleration of the decay rate is so slow at 1440 minutes that further loss of resistance can be expected to be small. It appears then, that for the initial mortality response there is a "permanent"* increase in resistance.

A "permanent" resistive process independent of the transient phenomenon is also tentatively indicated in results from a preliminary series of split-dose exposures of 6-day chick embryos.⁽²⁾ The effectiveness of the challenging dose decreased as the period between exposures was increased up to 360 minutes, but with intervals between 360 and 1440 minutes there was no further change in radiosensitivity. The initial mortality response was equivalent to that expected if the effect of the initial exposure had been reversed and a small amount of resistance developed. There was no evidence of a reduced radiosensitivity comparable in magnitude to the transient resistance observed in the 3-day chick.

A diagrammatic summary of the reversal and resistive processes that can be inferred from these experiments is shown in Figure 22. The lethal bound is indicated by the level of radiosensitivity observed in the untreated chick, equal to about 900 r of γ -rays for 50% mortality in the initial period. As time after the initial exposure is increased, the additional dose required to reach the lethal bound increases. For the first 110 minutes a linear reversal is the action of significance; but after 110 minutes, both transient and permanent induced resistive processes govern the decrease in radiosensitivity. This type of illustrative plan to represent formal recovery based on data from mammalian species has been presented by Sacher.⁽³⁾

Most provocative is the demonstration that resistive processes operate during as well as after irradiation. A resistance is not revealed with a single protracted exposure but can be demonstrated when a protracted dose is immediately followed by a high-rate challenging exposure. These results suggest that the action underlying the resistance phenomenon may be a depletion of some element that determines the sensitivity of the

*"Permanent" is used here in a relative sense to differentiate this process from the transient resistance that is lost in a matter of hours. No tests have been carried out to determine the presence of a decreased level of radiosensitivity after weeks or months.

organism to the initial mortality response. If it is postulated that for mortality in this period the first lethal event occurs when the animal exceeds its innate lethal bound, then manifestations of an induced resistance can only be appraised for the survivors of the more primary effect.

Figure 22. Action of reversal and resistive processes with respect to intervention time through 1440 min after an initial dose of 700 r, Co^{60} γ -rays. Vertical bars represent additional dose required for equivalent effect (50% mortality). Reversal of first dose (...). Early actions of permanent resistance postulated.

The induced resistance to the initial radiation response is functionally related to the size of initiating dose and the time available for its development. Interpretation of the resistive phenomenon has been considered in terms of alteration in radiosensitivity of a cell system. In view of what is known about the initial syndrome and the time required for its expression, a more probable hypothesis may be the temporary elimination of some essential subcellular factor. Restoration of sensitivity toward the normal level may then reflect a reconstruction.

The development of the resistive state in the irradiated chick indicates that for the initial mortality response, a decrease in dose effectiveness cannot be considered merely as recovery of induced injury. Thus, in multiple exposures to ionizing radiations, each response may alter the physiological state of the organism and thereby alter the response to a subsequent exposure. For this reason, a hypothesis based solely on the assumption of additivity of effects may lead to erroneous estimates of recovery rates.

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THE CELL GENERATION RATE DISTRIBUTION

Herbert E. Kubitschek

A study of cell generation times in E. coli was reported previously.⁽¹⁾ A critical reinterpretation of these results has been made possible by examining the distribution of the reciprocal of cell generation time, termed generation rate, and having units of rate of division per unit time.

Figure 23 shows a histogram of the generation rates observed for E. coli in four microcolonies.⁽¹⁾ Division was recorded as the time of appearance of a septum between daughter cells. The normal distribution is a good approximation to these data, shown by plotting the cumulative distribution of the same data on arithmetic probability paper (Figure 24).

Figure 23

The distribution of generation rates of E. coli B/r.

Figure 24
The cumulative distribution of generation rates for E. coli B/r.

Cell generation rates appear to be distributed normally for several other extremely different kinds of cells, including a yeast, a protozoan, and a strain of human tissue culture. Cumulative generation rate distributions

for these other cell types were constructed from published generation time histograms. Figure 25 shows the generation rate distributions for the diploid yeast Saccharomyces cerevisiae at three different temperatures, from data of Burns,⁽²⁾ who measured the appearance time of buds; Figure 26, the distribution for Tetrahymena geleii HS, from data of Prescott,⁽³⁾ who recorded completion of cytoplasmic fission; and Figure 27, the distribution for HeLa cells, data of Hsu,⁽⁴⁾ who recorded from anaphase to anaphase. The observation that the normal distribution seems to fit the generation rate distribution of each of these widely divergent cell types, with a different criterion for the time of fission in each case, leads to the hypothesis that the normal distribution is representative of the generation rate distribution in most cell cultures.

Figure 25

The cumulative distribution of generation rates for a diploid yeast at three different temperatures. Saccharomyces cerevisiae, data of Burns.

Figure 26

The cumulative distribution of generation rates for Tetrahymena geleii. Data from Prescott.

For E. coli, considering the total number of 168 cell generation times which were observed, mother and daughter generation rates appear uncorrelated ($r \sim +0.10$, 160 mother-daughter pairs), but sister generation

rates are highly correlated ($r \sim +0.44$, 84 sister pairs, $p < 0.01$). A closer examination of the data reveals that a significant negative correlation exists between the generation rates of the most rapidly dividing daughters and their mothers. For the 11 daughters which divided most rapidly, coming from 9 mother cells, only one mother cell divided more rapidly than average.

Figure 27

The cumulative distribution of generation rates for HeLa cells. Data from Hsu.

The sister-sister correlation indicates that an inherited factor tends to make sister cell generation rates similar. This similarity can hardly be genetic since mutation rates in *E. coli* are minute, approximately 10^{-7} to 10^{-8} per hour per bacterium. The lack of a positive mother-daughter correlation might imply (a) that inherited differences are rapidly compensated for by "feedback" mechanisms, or (b) that the daughter cells carry no "memory" of the processes leading to cell division in the mother cell; if cell division is dependent upon the accumulation of specific intermediates, these might be destroyed during cell division, itself. Some evidence for (a) is provided by the negative correlation between the generation rates of the most rapidly dividing daughters and their mothers.

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INFECTIVE ORGANISMS LIVING IN THE CYTOPLASM OF AMOEBA PROTEUS

E. W. Daniels and L. E. Roth

Evidence from electron and phase microscopy shows that minute organisms are present in the cytoplasm of Amoeba proteus, a common nonparasitic amoeba. These minute organisms live in vesicles within the cytoplasm (Figure 28). Food animals captured by the amoeba are immobilized and partly digested within a day, but the organisms described in this report remain active after the amoebae have been starved for more than two weeks and washed repeatedly in sterile water. The functional relationship between these minute organisms and the host amoebae (infective, parasitic, symbiotic, etc.) is unknown.

The findings presented here are discussed in regard to autoradiographic studies of other investigators on A. proteus. Occasional reports of deoxyribose nucleic acid (DNA) synthesis in the cytoplasm can be explained, and some of the controversy concerning nuclear versus cytoplasmic sites of ribose nucleic acid synthesis (RNA) can be better understood as a result of these investigations.

Experimental Procedure

Most of the amoebae used for these studies were obtained from the General Biological Supply House, Inc., in Chicago in 1957 and in 1960. They were cultured in this laboratory in glass-distilled water and fed axenically grown ciliates, Tetrahymena pyriformis (Strain W), which has no infecting organisms. The amoebae were fixed in 1% osmium tetroxide buffered to pH 8.0 with veronal acetate, dehydrated in a graded ethanol series and embedded in methacrylate. Sections were cut at 75m μ settings of the Porter-Blum Microtome and examined in an RCA EMU 3E electron microscope. Phase-contrast observations were made with a Zeiss Winkel microscope at a magnification of 500 diameters both on flattened, living amoebae and on amoebae immediately after rupturing. Some of the amoebae were starved for 7 days, thoroughly washed in sterile medium, and fed for two weeks on axenically grown Tetrahymena. The amoebae were then divided into four groups of 26 each; one group served as a nonirradiated control, and the others were subjected to γ -irradiation (Co⁶⁰ source) totaling 100, 200, or 300 kr at the rate of 5000 r/min. Following exposure, each amoeba was placed in a separate depression cup, fed, and observed for death or the establishment of a clone.

Figure 28. Sections through vesicles (V) in the cytoplasm of *Amoeba proteus*.

A. spheroidal vesicle ($2\mu \times 3\mu$) containing bacteria-like organisms; (M) mitochondria; (G) groundplasm; B. section through a spirilized organism within a vesicle; C. section through a coiled form that apparently had about 3.5 turns at the time of fixation.

Results

Electron micrographs show that the cytoplasm of A. proteus contains vesicles of several different sizes and types, in addition to the food vacuoles and cytoplasmic organelle systems such as nuclei, mitochondria, Golgi and groundplasm. Many of these organism-containing vesicles are spheroidal with diameters consistently in the 3 to 5- μ range (Figure 28A). The bacteria-like organisms within them are extremely active in the living state. These vesicles are difficult to break open. A second type of vesicle, distinctly larger and measuring about 10 to 12 μ in diameter, is spherical and contains a large number of very minute motile, curved rod forms about 0.2 μ by 1 μ . This vesicle ruptures very easily when pressure is placed on the amoeba. When the tiny forms are liberated, they are very difficult to see with the light microscope, even under optimum viewing conditions. Not more than one of these large vesicles has been found per amoeba, and many amoebae apparently contain none. On the other hand, nearly all of a hundred amoebae examined contained the smaller 3 to 5 μ vesicles. A third type of vesicle is variously elongated and contains a single, motile spirilized form with a few turns (Figure 28 B and C). Some of these are the size of mitochondria (1 to 2 μ), whereas others are somewhat larger. A fourth type is definitely elongated and contains a non-motile, tightly-coiled form with as many as 12 turns.

Our attention was turned next to methods of clearing the amoeba cytoplasm of the "infective" organisms. As previously mentioned, starvation was ineffective. Penicillin treatment was also ineffective. Since A. proteus is relatively radioresistant⁽¹⁾, ionizing radiation was also used in an attempt to free the cytoplasm of these minute organisms. Doses of 300 kr γ -radiation caused no deaths during exposure although no amoebae survived longer than 3 weeks. Following 200 kr γ -radiation, all except one amoeba died within 3 weeks. This survivor divided several times, but the daughter cells that were examined contained typical "infections." Of the amoebae irradiated with 100 kr γ -rays, 15 of 26 gave rise to clones, not significantly different from the proportion of clones produced by nonirradiated controls. Phase microscopic examination showed that all clones of the control group were infected; however, a definite reduction of infection was observed in 3 surviving clones in the 100-kr group. These clones all died during the next several weeks.

Discussion

Although the synthesis of DNA is commonly associated only with the nucleus, occasional reports present evidence for cytoplasmic DNA synthesis in addition to nuclear synthesis. Such a case in A. proteus was reported by Plaut and Sagan,⁽²⁾ who studied labeling by tritiated thymidine in organisms that were previously starved for 7 days and washed in a penicillin solution

immediately before incubation in the label-containing culture fluid. All of the amoebae showed radioactivity in the cytoplasm after 44 hr of incubation, while few showed it in the nucleus; ribonuclease treatment of sections before autoradiography had no effect on labeling, but deoxyribonuclease treatment resulted in essentially no demonstrable label. Therefore, they suggest that the labeled component is DNA and postulate that it is either synthesized by a normal cytoplasmic process or by a cytoplasmic infective agent. Similar findings were more recently reported by Plaut.⁽³⁾

Similarly, the literature contains conflicting reports on the synthesis of RNA in the cytoplasm of A. proteus. Prescott⁽⁴⁾ studied labeling by C¹⁴-uracil and reported that whole and nucleate halves showed cytoplasmic incorporation, while enucleate halves did not. A further report by Prescott⁽⁵⁾ shows no incorporation of C¹⁴-labeled uracil, orotic acid or adenine in enucleate halves. However, Plaut and Rustad⁽⁶⁾ have reported incorporation of adenine-8-C¹⁴ in enucleated halves of amoebae, and suggested a cytoplasmic process of incorporation of precursor(s) which operates in the absence of the nucleus. Therefore, they maintain that two processes exist, this cytoplasmic process and the nuclear incorporation process, but state that their relative magnitudes in intact cells are unknown. Further work by the same investigators⁽⁷⁾ using C¹⁴-uracil and C¹⁴-orotic acid indicates that both of these precursors were incorporated into cytoplasmic RNA in both nucleate and enucleate cells. Plaut and Rustad probably used a strain of A. proteus which harbored minute cytoplasmic organisms such as those described here, whereas Prescott probably had a strain of A. proteus which was free from these forms.

The present report is one result of a prolonged study of several species of amoebae both by electron microscopy of thin sections and by phase microscopy of living organisms. Very small bacteria-like organisms were found repeatedly in the cytoplasm of A. proteus by phase microscopy and are related to structures seen in numerous vesicles and in many electron microscope sections. Mercer⁽⁸⁾ pictures electron-dense structures which he calls unknown bodies in his electron microscopic study of another stock of A. proteus. It is possible that these are "infective" forms also. In a recent review, Branchet⁽⁹⁾ has postulated that bacteria resistant to digestion are phagocytized by the amoebae and that these account for RNA synthesis in the cytoplasm. What we are demonstrating in this report might be captured bacteria and allied forms that resist digestion, penicillin, and radiation. The evidence indicates that "infection" or "contamination" of amoeba cytoplasm is probably rather widespread. Pelomyxa illinoisensis, a radiosensitive giant amoeba, harbors electron-dense bodies that might be foreign organisms encased within vesicles.⁽¹⁰⁾ Bacteria that are symbiotic or parasitic in protozoa have been recently placed in an addendum to Class II, Schizomycetes in Bergey's Manual.⁽¹¹⁾ Paramecium aurelia also has bacteria-like organisms living in the cytoplasm.⁽¹²⁾

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ELECTRON MICROSCOPIC STUDY OF THE FINE STRUCTURE OF THE FAT CELL AS RELATED TO FUNCTION

F. Wassermann and Thomas F. McDonald

Examination of the fat cell with the electron microscope^(1,2) has not up to now yielded significant information on changes that could be justly correlated with function. This is quite understandable if we remember the complexity of the metabolism of the fat cell. Uptake of the constituents of fat and their recombination and storage on the one hand, and hydrolysis of the fat and release of the constituents on the other, are the two main functional phases of the fat cell. These not only succeed each other but may overlap in the same cell. Different cells in a given fat depot may be in different phases of activity.

To study the fine structure of the fat cell as related to function, one needs, therefore, a material in which the cells may be expected to be at least predominantly in one or the other of the two phases of activity, which will be referred to here as those of impletion and depletion.

In the mouse and the rat the inguinal fat body, whose embryonic development has been studied,⁽³⁾ although not inactive during prenatal time, does not begin to elaborate fat until immediately after birth. The fat cell taken from this depot on the first day of life will be, therefore, in the phase of impletion. If one removes such animals from the mother for periods of starvation, which may be extended to about 30 hours, one can be certain that the cells will be in a state of depletion. Returned to the mother after starvation, the suckling will immediately utilize the milk fat; hence the cells must be engaged in the storage of fat.

Accordingly, the inguinal fat bodies were excised (1) from term embryos, (2) from newborn animals (first to third day) whose stomachs and lacteals were well filled, (3) from starving 1- and 2-day-old animals, and (4) from animals of the same age that were returned for varying periods to the mothers before they were sacrificed. This report is limited mainly to our findings in the mouse although rat material was also used. The isolated fat body was immediately covered by osmic acid-bichromate fixative⁽⁴⁾ and cut into small pieces, which were then kept immersed in the fixative for 1 hr at room temperature. The dehydrating, embedding in methacrylate, and sectioning were done in the usual way. The electron microscope used was the RCA EMU 3A model.

The cells of the prenatal fat body show no specific characteristics as to cytoplasmic and nuclear structure, but are distinguished by the presence of larger or smaller vacuoles; one of medium size is seen in Figure 29. Although these vacuoles may sometimes contain much more gray material than that in Figure 29, the osmium-blackened enclosures characteristic of definite fat cells do not occur.

Figure 29. Part of a cell of the prenatal inguinal fat body of the mouse. Except for vacuoles containing a not yet defined material, the cell does not show special characteristics.
n-nucleus, m-mitochondria, v-vacuole.

During the first hours of postnatal life the picture of the fat cell changes rapidly in response to the absorbed milk fat. Now, one finds in each cell several small and larger, deeply blackened enclosures (Figure 30), some of which are not completely rounded as are the fat droplets in older fat cells. Smaller droplets are fusing or are taken into larger ones already formed.

Figure 30. Part of a fat cell of mouse on the first day post partum. Notice the osmium-blackened fat droplets of various sizes (fd), extremely swollen mitochondria (m), and masses of black granules dispersed throughout the cytoplasm.

These cells, which are certainly engaged in fat synthesis, differ from the prenatal cells, aside from the presence of fat droplets, mainly in two ways: first, the mitochondria are more or less enlarged; secondly, fine black granules in varying amounts are dispersed randomly throughout the cytoplasm.

The mitochondria appear to swell during impletion so that, as in the cell of a three-day-old mouse (Figure 31), the cristae are widely separated and the intervening space is filled by threads of a material somewhat denser than the background. In the cell taken from animals on the first day (Figure 30) when impletion is probably most intense, the inner structure of the mitochondria has almost entirely vanished. The membrane, however, is still very distinct and allows identification of the three mitochondria at the right in the picture.

Figure 31. Part of fat cell of 3-day-old mouse. Notice the fat droplets (fd), the swollen mitochondria (m) and the black granules.

As the representative cells in Figures 30 and 32 show, there is considerable variation in appearance of the mitochondria among animals of the same age groups. The same is true of the black granules, which in the cell of Figure 30 are present in much greater amounts than in that of Figures 31 and 32. As a rule the granules are not seen inside the mitochondria, but this is not without exception as Figure 31 shows. It may well be that granules are displaced either during fixation or sectioning.

Figure 32. Part of fat cell of a mouse on the first postnatal day. The mitochondria (m) are less swollen than those in the cells of Figures 30 and 31. Adjacent to the cell is a capillary (cap) with erythrocyte (er). Notice black granules in the fat cell and chylomicra in the capillary.

Following the ingestion of milk, chylomicra appear in the blood capillaries (Figure 32). With the electron microscope their relationship to the capillary wall can be observed. We shall come back to this point in the discussion of our findings.

In animals that had been nursed for several hours on the first day of postnatal life and then kept away from their mothers for periods of 8 to 30 hours, the majority of the fat cells were markedly different from those in well-nourished animals. The difference concerns mainly the mitochondria (Figure 33), which are now of the usual size and structure or sometimes unusually compact and dark so that the inner structure can no longer be clearly seen.

Figure 33. Parts of two fat cells from a mouse kept away from the mother for 8-1/2 hours before sacrificing. Notice the dark compact mitochondria (m), the fat droplets (fd) and the black granules.

The fat droplets often have irregular surfaces giving the impression that their material is melting away. Tiny black granules are dispersed throughout the cytoplasm and sometimes seem to radiate out from the fat droplets. The small dark granules in the depleting cells are not different from those appearing in the cells of animals of the same age during impletion; in both phases of activity they vary widely in amount, size and degree of clustering.

The two cells in Figure 34 belong to the fat body of an animal that had been properly nursed by the mother for two days and then kept away from the mother for $9\frac{1}{2}$ hours before fixation of the tissue. The cell at the right in the picture shows all the aforesaid criteria of the depleting cell. However, the cell at the left with washed-out mitochondria and rather coarse granules has the appearance described above as characteristic of the cell during impletion. Such pictures suggest that adjacent fat cells may behave independently and may at a given moment differ in their respective activities. It is conceivable that in an animal richly supplied with fat one or another cell may not respond to starvation as readily as its neighbors.

Figure 34. Sections of two fat cells from a 2-day-old mouse starved for 9-1/2 hours before sacrificing. Notice the differences in mitochondria (m) and granules between the two cells (see text).

The independence of the individual cells is further underscored by the fact that in animals returned to the mothers after a starvation period, not all of the cells of the same fat lobule show a complete reversal from depletion to impletion. Figure 35 shows part of the cytoplasm of a cell which after a refeeding time of only $3\frac{1}{2}$ hours contains mitochondria that are already considerably swollen; the black granules, however, were missing in this case.

Figure 35. Part of fat cell of a 1-day-old mouse refed by the mother after 8-1/2 hours starvation. Note the swollen mitochondria (m). The black granules seen in Figures 30, 31, 33, 34 are here very scarce if present at all. Notice the close relationship between an apparently forming fat droplet and the adjacent swollen mitochondrion. (arrow).

That they can be present after two hours' feeding following a starvation period of 7 hours is seen in the cell of Figure 36.

Figure 36. Part of fat cell of a 2-day-old mouse that was refed by the mother after 7 hours starvation. Notice the great number of black granules in this case. Adjacent to the cell a blood capillary (cap) with chylomicra at the wall of the lumen.

In the capillary adjacent to the fat cell of Figure 36 the already demonstrated chylomicra can be seen. None are seen within the endothelial cells nor are the black granules seen in the fat cell present in the endothelial cells.

Discussion

In cells from the inguinal fat body of the mouse on the first day of postnatal life the mitochondria were considerably enlarged as compared with those of cells in the prenatal fat body. Mitochondria were also swollen, and their inner structure was more or less lost, in the cells in which fat storage was resumed after a period of cessation of fat synthesis while the animal was starved. Apparently, the increase in volume of the mitochondria leads to an unfolding of the cristae which become more or less completely incorporated in the inner membrane of the mitochondrion. We do not know what this reorientation of the mitochondrial lamellar system may mean. It seems to be related to the impletion process since in the animals that were taken from the mother, the mitochondria, which must have been swollen before, are in a compact state and show the usual cristae unless they are of an unusually high density with consequent obscuring of the inner structure.

In both the impletion and the depletion phases the cytoplasm of the fat cell was found to contain submicroscopic granules that are deep black (osmiophilic). They are clearly different from the rather uniform somewhat larger nonosmiophilic granules most clearly seen in Figure 35, which are of the nature of RNA granules. The black granules, whether they represent the precipitate of a material that was dissolved in the living cell, or whether they correspond to a preexistent particulate material, are certainly specific components of the fat cell cytoplasm. They are not present in the cytoplasm of adjacent blood capillaries (see Figure 36). The relationship of the small granules to the fat droplets is especially obvious in the depleting cell, where they seem to radiate from the surface of the fat droplets as seen in Figure 33. The electron microscopic pictures do not allow any statement about the nature of the black granules, which may be minute fat droplets or particles consisting of fatty acids possibly combined with protein. All we can say, in agreement with Chase,⁽²⁾ is that this material is on the way either into or out of the fat cell. It is incorporated into the fat droplets during impletion, and it derives from them during depletion.

The electron microscope enables us to add a few more data pertinent to the problem of transport of fat from the blood to the fat depots or vice versa. The chylomicra in the blood capillaries on the one side and the black granules on the other are the two demonstrable states of the material during its transport. Between these two states the material cannot be seen with the electron microscope.

The chylomicra, consisting according to Frazer and coworkers⁽⁵⁾ of a combination of fat and protein bounded by a thin film of lecithin, were mostly found attached to the inner surface of the capillary wall, sometimes lying in indentations of the endothelial cells. Rarely, as shown in Figure 37 they seemed to be enclosed in the endothelial cell, but whether they were actually surrounded by cytoplasm could not be made out with certainty.

Figure 37. Blood capillary in an impleting fat depot with erythrocyte (er) and numerous chylomicra at the wall of the lumen. A group of chylomicra gives the impression of being surrounded by cytoplasm (arrow) but otherwise the endothelial cells are free of fat droplets.

As a rule, no chylomicra or osmiophilic droplets that might be derived from them, nor black granules, as in the fat cells, were seen within the cytoplasm of the endothelium. The surface structures of the endothelial cell at the outside of the capillary wall also were always free of such material, as was the tissue between the capillaries and the fat cells. We were especially interested to find out whether in the surface structures of the fat cell, commonly referred to as the basement membrane, (see Wassermann and McDonald⁽⁶⁾) fat droplets or particles like the black granules could be observed. This was not the case. The black granules

occurred only within the fat cell, and plasma membrane is, therefore, the dividing line which separates the area of the black granules from the homogeneous material that covers the cell (Figure 38).

Figure 38. Two fat cells, separated by a blood capillary (cap), from a 2-day-old mouse. Notice the fat droplets (fd), the swollen mitochondria (m) and the black granules. The picture shows how sharply the black granules are confined to the cytoplasm of the fat cell.

We conclude from these observations that the material of the chylomicra permeates the membrane of the endothelial cells in a form, probably that of the constituents of fat, which cannot be seen with the electron microscope; in other words, the endothelial cells are probably actively involved in the transport. Furthermore, we can say that within the endothelial cell no changes occur that would render the material visible as black granules. The same holds for the passage of the material through the extracellular surface structures of both the endothelial and the fat cells. Inside the fat cell, however, (probably in the plasma membrane) the change takes place which leads to the appearance or, in depletion, to the disappearance of the granules.

Thus, the electron microscopic observations show, at present only in general terms, the possibility of localizing within the pathway of fat from the blood to the fat cell some of the steps in the series of chemical changes which are involved in the transport. The mitochondrial changes described in this report suggest that these organelles are involved in both impletion and depletion of the fat cell but in different ways. Whether this means an active participation cannot be told at present. The electron microscopic studies will be continued and will be complemented by cytochemical investigations of the fat cell.

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ATTEMPTS AT ENZYMATIC INDUCTION OF E. COLI

Robert N. Feinstein*

Several experiments were performed to test the following concept of the mechanism of the process of radiation induction of bacteria:

1. The conversion of prophage to active phage is normally enzymatic, perhaps comparable to the enzymatic conversion of phosphorylase a to phosphorylase b.
2. The converting (activating) enzyme is normally held in check by a co-existing inhibitor.
3. The inhibitor is labile towards UV or ionizing radiation.
4. When an inducible organism is irradiated, the inhibitor is destroyed; "converting enzyme" activity is thereupon released; and so prophage is converted to active phage.

Experiments were designed to test the possibility of destroying the inhibitor enzymatically (by RNase if it were an RNA, by DNase if it were a DNA, by proteolytic enzymes if it were a protein, etc.).

Two important experimental difficulties must be borne in mind: First, if one tests induction in intact cells, it is extremely unlikely that such enzymes as RNase, DNase, or the proteolytic enzymes will ever penetrate the cell. A device which may avoid this difficulty is the use of protoplasts, which have been shown by Borek and Ryan⁽¹⁾ to be inducible by radiation in certain cases. Second, the possibility must be considered that, while the inhibitor may be more radiation-sensitive than the "converting enzyme," it may be no more sensitive than the enzyme to proteolytic treatment. That is to say, treatment with proteolytic enzymes may destroy both the "converting enzyme" and its inhibitor, thus negating the entire concept.

It is thus evident that while positive results would be of considerable interest, negative results would be of no significance and would not destroy the concept described above.

Three experiments have been performed in all.

a) Intact E. coli cells were treated with DNase, with RNase, and with a mixture of DNase and RNase (100 μ g of each enzyme per 2 ml of cell suspension).

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

(b) Protoplasts of E. coli were prepared by the technique of Fraser and Mahler⁽²⁾. These were then treated with DNase, RNase, carboxypeptidase, or rat kidney extract (as source of catheptic enzymes).

(c) Repeat of (b), but allowing more time for protoplasting, and with more carefully timed and executed enzyme preparations. The kidney preparation, in particular, was carefully prepared, finally being centrifuged to remove the carboxypeptidase inhibitor, which is known to precipitate under the circumstances.

Results were uniformly negative. There was no slightest indication of more phage formation in the treated protoplasts than in the control preparation. Experiments were therefore halted.

I wish to record here my appreciation to Dr. H. Marcovich, who advised on the microbiological aspects of this concept and who performed the bulk of the microbiological portions of the experiments described.

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INTRANSIGENCIES IN THE STUDY OF CHOLESTEROL ESTER METABOLISM

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We have previously reported the development of a method which permits the isolation of individual cholesterol esters in an intact condition.⁽¹⁾ This permits one to measure the specific activity of the cholesterol portion of the ester and to compare the specific activities of the sterol in esters of different fatty acids. When such measurements are made thirty minutes after the injection of labeled acetate, these activities are heterogeneous, while four hours after isotope administration they are homogeneous.⁽²⁾ Similarly, when labeled cholesterol is fed at hourly intervals for seven days, it can be shown that the liver cholesterol esters exhibit heterogeneity in their activities, and that the order of specific activities changes during the feeding period.⁽³⁾

It seemed fruitful to us to investigate the turnover of cholesterol esters in liver and plasma of normal and irradiated animals and such a study was carried out. In the course of these experiments, it became evident that cholesterol ester metabolism in the rat does not fulfill certain conditions necessary for kinetic studies. These conditions, their significance in cholesterol metabolism, and their implication in the further study of this aspect of cholesterol metabolism are the subjects of this report.

In the design of studies intended to determine rates of synthesis, secretion, transport and conversion of a metabolite in the intact animal, the tacit assumption is usually made that during the measurement, the system is in a steady state. In reality such an assumption is rarely, if ever, justified. The experimenter further assumes, until it is proven otherwise, that under identical conditions of age, strain, sex, diet, housing and time of day, one animal is equivalent to another. Finally (and this is hardly ever consciously recognized) it is assumed that the act of making the measurement has no effect on the results obtained. The sequence of experiments described below was designed on this basis; the results indicate that each assumption is singularly untenable in studies of cholesterol ester metabolism.

Experimental

Animals. Groups of four male Sprague-Dawley rats, fed a stock diet and weighing 250 to 350 g, were given individual intraperitoneal injections of 100 μ c of sodium acetate-1-C¹⁴ and were killed after 0.5, 1, 2, 4, or 12 hours by cardiac puncture and exsanguination. All injections were performed between 9 and 10 A.M, with the exception of the 12-hour incubation

which was started at 9 P.M. The plasmas in each group were pooled for analysis. The livers were removed, and after extraction of the lipid, aliquots from each extract were pooled for ester analysis and determination of specific activities.

Analytical. All procedures used in the extraction of lipid and in the isolation, fractionation and analysis of cholesterol esters have been described previously.⁽¹⁻³⁾ In the present study, selection of the samples of cholesterol esters for hydrolysis and precipitation was done in a slightly different fashion than in the previous experiments. Instead of taking sequential samples within each peak and treating them individually, the same samples were pooled and analysed in triplicate. While this procedure reduced the number of analyses in each fraction and hence, the number available for statistical comparison, it tended in most cases to increase the precision of the results.

The composition of the plasma and liver esters at each time interval is shown in Table 15. With the possible exception of the first value for saturated esters in plasma, these values appeared to be quite constant during the intervals studied and were in good agreement with values obtained in earlier studies.

TABLE 15

Composition of plasma and liver esters obtained
during turnover studies

Percent composition of esters					
	Time after injection of sodium acetate-1-C ¹⁴				
	0.5 hr	1.0 hr	2.0 hr	4.0 hr	12.0 hr
Plasma					
Saturated	3.5	6.6	11.0	9.9	-
Oleate	8.2	10.8	12.1	12.3	-
Linoleate	40.6	26.4	28.8	26.2	-
Arachidonate	47.7	56.2	51.5	51.5	-
Liver					
Saturated	19.3	14.2	10.5	15.1	14.0
Oleate	26.0	33.4	33.3	31.8	37.2
Linoleate	33.4	31.6	36.1	29.2	35.4
Arachidonate	21.3	20.9	20.0	23.8	13.4

Table 16 lists the specific activities of these esters at various periods after the injection of acetate, as percentages of the liver free cholesterol specific activities which are listed at the top of each column. This method of presentation was chosen because it was considered to represent an internal standard suitable for use in the comparison of groups and independent of group-to-group variations.

TABLE 16
Specific activity of cholesterol in various cholesterol esters
at various times after injection of sodium acetate-1-C¹⁴

Specific activity (% of liver free cholesterol activity)										
	Time after injection of sodium acetate-1-C ¹⁴									
	0 5 hr (12,610)*		1 0 hr (5,365)*		2 0 hr (4,636)*		4 0 hr (7,350)*		12 0 hr (745)*	
Plasma										
Saturated	24 4	(2 4)**	17 5	(3 6)	14 8	(2 7)	17 7	(2 3)	670	(134)
Oleate	21 8	(0 0)	28 1	(7 7)	23 6	(4 4)	24 0	(2 0)	1165	(53)
Linoleate	7 3	(0 4)	13 9	(1 9)	20 7	(0 3)	34 2	(3 7)	1033	(17)
Arachidonate	6.6	(0 2)	8 5	(0 4)	16 5	(1 9)	29 1	(3 4)	1316	(59)
p [†]	0 01		0 1 > p > 0 05		0 1 > p > 0 05		0 05 > p > 0 01		0 05 > p > 0 01	
Liver										
Saturated	14 8	(1 7)	32 5	(6 8)	21 8	(2 4)	39 9	(2 6)	964	(44)
Oleate	23 4	(0 4)	29 4	(0 4)	43 9	(1 9)	53 4	(4 1)	1023	(38)
Linoleate	20 2	(0 5)	25 6	(0 7)	42 0	(1 4)	45 6	(2 6)	1004	(25)
p [†]	0 01		0 01		0 01		0 01		0 25	

*Specific activity of the liver free cholesterol activity, dpm/mg

**Standard error of the mean

†Probability of homogeneity calculated from a one-way analysis of variance

The probability of homogeneity at each time point was calculated and is shown below each set of values. At all time intervals, the plasma values were heterogeneous while the liver specific activities achieved homogeneity sometime after 4 hours. Examination of the specific activities of cholesterol linoleate and arachidonate in both liver and plasma showed a gradual and progressive rise and, until at least 4 hours had elapsed, the plasma esters showed a lower specific activity than the corresponding liver esters. On the other hand, at early time periods, the activities of saturated and oleate esters of plasma were higher than, or equal to, those of liver and then dropped below them.

The ratio of plasma to liver specific activity was plotted versus time for each of the four fractions and is shown in Figure 39. The values for cholesterol linoleate and arachidonate each form a curve which is convex in an upward direction, supporting the concept that these esters are of hepatic origin. In contrast, the values for saturated and oleate esters fall on a descending curve, which suggested that these esters in plasma may not have their origin in the liver.

Figure 39

Ratio of plasma specific activity to liver specific activity as a function of time, for saturated, oleate, linoleate and arachidonate esters of cholesterol.

In the course of subsequent experiments we had occasion to re-check the specific activities of the plasma and liver esters 30 minutes after the injection of acetate and, to our dismay, found that the specific activity of the plasma ester relative to that of the liver ester behaved in a capricious manner. This necessitated a close scrutiny of (a) the analytical procedures and (b) the physiological status of the animals.

Despite the inherently useful features of our techniques for measuring cholesterol incorporation into esterified forms, they suffer from the disadvantage of being too cumbersome to apply to large numbers of samples. Therefore, it was necessary to assess the limits of reproducibility of the method itself, as well as the variation in a normal population.

We have previously reported the reproducibility of the ester fractionation procedure based on the analysis, in triplicate, of a sample of human plasma esters.⁽¹⁾ This showed the variation to be 3% of the value for large components, increasing to 16% for constituents comprising less than 10%. Such a comparison does not, however, take into account possible differences in the extraction and separation of the sterol esters prior to their fractionation. Therefore, six animals were killed and the livers and plasmas each pooled and divided into two groups. All extractions, separations and fractionations were carried out in parallel; the comparison of compositions obtained is shown in Table 17. The data indicate that the procedures leading to the fractionation are not a significant source of error. Occasionally the fractionation of liver esters was interrupted before the arachidonate peak had been eluted, necessitating a re-run of the

chromatogram to obtain the specific activity of the cholesterol in this ester. This provided an opportunity to compare the reproducibility of the specific activities of the esters obtained during fractionation (Table 17).

TABLE 17

Reproducibility of ester fractionation from a pooled source and of specific activity measurements of the cholesterol obtained from ester fractions of liver

Ester fractionation of pooled plasma and pooled liver samples				
	Fatty acid composition of plasma sterol esters, %		Fatty acid composition of liver sterol esters, %	
	Run 1	Run 2	Run 1	Run 2
Saturated	9.2	11.5	9.7	14.1
Oleate	12.8	11.8	41.7	39.0
Linoleate	37.5	36.5	37.6	34.3
Arachidonate	40.2	40.2	11.0	12.4

Specific activity of cholesterol esters of liver, dpm/mg				
	Sample I		Sample II	
	Run 1	Run 2	Run 1	Run 2
Saturated	1148	1237	4236	3473
Oleate	1389	1353	5389	5189
Linoleate	1658	1465	5269	5263
Arachidonate	-	1106	-	1952

Table 18 summarizes data obtained from 6 groups of normal animals; each group consisted of 4 animals. All groups received labeled acetate and were killed 30 minutes later. From the mean value, standard error and range of compositions in liver and plasma esters, it would appear that the composition of cholesterol esters shows a greater variation than would be attributable to analytical errors. The greatest range of values was found to take place in the percent of saturated and oleate esters of plasma; the corresponding esters in liver varied within much closer limits. The rates of incorporation of newly synthesized cholesterol into

these esters showed similar fluctuations, even when the known variations of cholesterol synthesis are taken into account. Again, the variations in the saturated and oleate esters of plasma were noticeably greater than all other esters.

TABLE 18

Average composition of plasma and liver esters in pooled samples and the incorporation of newly synthesized cholesterol into these esters

Ester composition						
	Plasma			Liver		
	%	S.E.*	Range	%	S.E.*	Range
Saturated	10.9	2.0	(3.5-18.4)	14.0	1.6	(8.9-19.3)
Oleate	10.8	1.9	(4.9-15.7)	33.8	2.7	(26.0-45.0)
Linoleate	30.9	3.9	(18.0-40.6)	36.7	1.9	(29.5-41.5)
Arachidonate	47.5	5.5	(37.4-66.9)	15.5	1.7	(9.0-21.3)

Specific activity of ester cholesterol 30 min
after the injection of acetate**

	Plasma		Liver	
	%	S.E.*	%	S.E.*
Saturated	12.7	(3.4)	16.1	(1.7)
Oleate	12.8	(3.4)	20.8	(2.7)
Linoleate	7.3	(1.0)	19.5	(2.0)
Arachidonate	7.5	(1.6)	12.3	(2.3)

*Standard error of the mean, n equals 6.

**All values are in percent of liver free cholesterol specific activity.

Physiological Status Studies. The influence of physiological status, particularly of feeding pattern, was considered in several experiments of which two examples are reported here. In the first experiment, cognizance was taken of the nocturnal feeding habits of rats; a group of four animals was injected at 4 A.M. and killed 30 minutes later. In the second experiment, the constant-feeding technique^(3,4) was used. Three rats were fed their daily rations in hourly increments for a period of 10 days and then

removed just prior to the actual time of injection. The compositions and specific activities of the esters in the two groups are shown in Table 19. While the composition of the esters found in the animals injected at 4 A.M. was quite comparable to the "normal" controls, and all plasma values were well below the corresponding liver values, there was no significant heterogeneity of specific activity within either the plasma or liver esters. On the other hand, in the animals which had been fed at hourly intervals, the plasma saturated ester had a higher specific activity than the liver ester, and the incorporation of cholesterol into cholesterol arachidonate (usually possessing the lowest specific activity) was two- to three-fold greater than in the other esters.

TABLE 19

The effect of time of injection and of constant-feeding regime on the composition of cholesterol esters and the specific activity of cholesterol in these esters

Ester	Plasma		Liver	
	Composition, %	Specific activity, % liver free cholesterol*	Composition, %	Specific activity, % liver free cholesterol*
Experiment I. Four animals were injected at 4 A.M. and sacrificed 30 min later. Control values to which these should be compared are given in Table 18.				
Saturated	10.7	7.8 (0.4)**	10.2	18.0 (0.5)**
Oleate	13.6	9.8 (0.5)	37.4	17.9 (0.3)
Linoleate	35.4	9.6 (0.7)	38.3	19.3 (1.1)
Arachidonate	40.3	9.7 (0.3)	14.1	16.3 (0.4)
Probability of homogeneity†		-‡		-‡
Experiment II. Three animals were fed at hourly intervals for 10 days. They were injected at 9 A.M. just after the last portion of food had been consumed and were sacrificed 30 min later.				
Saturated	11.4	11.3 (0.7)	13.2	10.5 (2.2)
Oleate	23.0	11.9 (1.2)	49.6	13.1 (1.7)
Linoleate	21.9	11.8 (2.3)	27.9	16.0 (1.2)
Arachidonate	43.8	6.1 (0.6)	9.3	32.9 (1.9)
Probability of homogeneity†		0.05		<0.001

*The liver free cholesterol specific activities were 19,200 dpm/mg in Experiment I and 5458 dpm/mg in Experiment II.

**Standard error of the mean

†Calculated from a one-way analysis of variance.

‡These samples did not differ from a homogeneous population when measured at the 5% critical level of Snedecor's "F" test.

Reaction of Individual Animals to Experimental Conditions. At the time the foregoing experiments were performed, the sample requirements precluded the investigation of cholesterol ester metabolism in a single animal. The possibility that artifacts were being created by pooling samples could not be ruled out unless individual determinations were done. Therefore, efforts were made to reduce the sample size requirement and by a series of stringent economies, the methods were adapted to obtain the composition and specific activities of cholesterol esters in the liver and plasma of individual animals.

At the same time, reflection on the capricious behavior of the cholesterol esters suggested that the rates of esterification of liver cholesterol and that of other sites, and the subsequent rates of transfer to the plasma, might be changing within the relatively short incubation period of thirty minutes. A fast response of this nature in cholesterol ester metabolism, and its variation from animal to animal, seemed explainable only in terms of a nervous or adrenal reaction to the stress of unfamiliar surroundings, handling, injection and laboratory noise.

Accordingly, a "tranquilizing program" was begun on a group of four rats. The animals were brought to the laboratory every day for ten days and each individual received 15 minutes of handling, i.e., being stroked, picked up, turned over as if for injection, and so forth. On the day of injection, the animals were secluded in a quiet office adjacent to the laboratory; new sharp needles were used for the injections, which were performed in silence and with as little handling as possible. The animals were maintained in an undisturbed atmosphere during the incubation and were individually removed from the room 30 seconds before being killed by ether anaesthesia. The "stressed" rats were brought to the laboratory for the first time on the day of use and were placed in restraining cages just prior to injection. They were kept in this restraint for 30 minutes and then killed. Each liver and plasma sample was fractionated separately.

Table 20 lists the composition of liver and plasma esters in both groups. The liver esters of both groups were in remarkably close agreement; the plasma level of saturated esters in the "stressed" animals showed a small but significant drop. On the other hand, the specific activities of the liver esters (shown in Table 21) showed marked differences between the two groups. The "stressed" values displayed the familiar order of descending activities; oleate, linoleate, saturated and arachidonate ester. In the "tranquil" animals, the order was now: saturated, linoleate, oleate and arachidonate ester, and the absolute percentage of liver free cholesterol activity in the saturated and arachidonate esters was significantly higher than in these esters in the "stressed" animal.

TABLE 20

Composition of liver and plasma cholesterol esters in "tranquil"
and "stressed" rats

	Composition of esters, %	
	"Tranquil"	"Stressed"
Liver		
Saturated	13.1 (0.5)*	13.6 (0.9)
Oleate	34.3 (1.2)	36.8 (1.8)
Linoleate	31.7 (1.9)	29.8 (2.0)
Arachidonate	20.7 (2.6)	20.4 (2.8)
Plasma		
Saturated	11.8 (0.6)**	9.7 (0.2)**
Oleate	9.2 (1.3)	9.8 (1.0)
Linoleate	27.4 (0.9)	27.0 (1.0)
Arachidonate	51.4 (2.5)	53.4 (2.1)

*Standard error of the mean.

**Difference is significant at $p = 0.02$.

TABLE 21

Incorporation rates of newly-synthesized cholesterol into liver and
plasma esters of "tranquil" and "stressed" rats

	Percent of liver free cho- lesterol specific activity		Probability, p, of similarity using "t" test
	"Tranquil"	"Stressed"	
Liver			
Saturated	21.3 (2.2)*	10.9 (1.2)	<0.01
Oleate	16.2 (0.6)	15.2 (2.2)	ns**
Linoleate	20.8 (1.3)	13.7 (1.9)	ns
Arachidonate	12.6 (2.0)	6.0 (0.6)	0.02
Plasma			
Saturated	2.2 (0.8)	3.8 (1.4)	ns
Oleate	2.4 (1.0)	5.2 (1.8)	ns
Linoleate	2.4 (0.9)	3.5 (0.3)	ns
Arachidonate	3.4 (0.6)	3.7 (0.4)	ns

*Standard error of the mean.

**Not significant.

The specific activities of the plasma esters in the "stressed" animals tended to be higher than those of the "tranquil" animals, although both groups were quite low and it was not possible to attribute statistical significance to the differences. The transfer rates (calculated as the ratio of plasma ester activity to liver ester activity) of the two groups reflect this difference to a greater degree; the saturated and arachidonate ester values in the "stressed" animals are significantly higher than in the "tranquil" ones (Table 22).

TABLE 22

Transfer rates of liver cholesterol esters into plasma in
"tranquil" and "stressed" rats*

	"Tranquil"	"Stressed"	Probability, p, of similarity using "t" test
Ester			
Saturated	0.104 (0.041)**	0.338 (0.089)	0.05
Oleate	0.146 (0.057)	0.333 (0.080)	ns†
Linoleate	0.147 (0.060)	0.267 (0.031)	ns
Arachidonate	0.301 (0.109)	0.613 (0.061)	0.05

*Calculated as the ratio of specific activity of the plasma ester to that of the liver ester 30 min after the injection of acetate.

**Standard error of the mean.

†Not significant.

Taken as a group, these experiments indicate but do not explain a previously unsuspected complexity in cholesterol ester metabolism. There seems no reason to doubt that the mere introduction of the animal into an unfamiliar surrounding is sufficient to alter the rates at which cholesterol is esterified to different fatty acids. That one need not use a restraining cage to produce these changes is evident from the fact that the usual pattern of specific activities in the liver esters of the majority of animals studied coincides with those of the "stressed" group. One cannot predict, in a retrospective manner, how many, if any, of the phenomena encountered in the turnover and physiological status studies can be accounted for by this stress reaction; certainly its existence undermines the validity of any conclusion which might be drawn from them. Some evidence exists that these phenomena may be present in addition to the stress reactions.⁽⁵⁾

The lability of cholesterol ester metabolism in the intact animal leads to two conclusions: the first concerning the mechanisms of heterogeneity in esterification, the second concerning further investigation of the problem. Ever since our discovery of heterogeneity of specific activities in the cholesterol esters, we have sought the mechanism responsible for these differences. From earlier considerations,^(2,3) it was evident that some sort of compartmentalization according to fatty acid must exist in the pathways leading to the intact ester. One must then choose between two alternative postulates: First, the pools of individual fatty acids are similar in size and replacement rate; then the diversity in incorporation rates must lie in the existence of separate cholesterol pools. Second, it could be imagined that a single pool of free cholesterol is esterified with fatty acids existing in separate pools with individually characteristic replacement rates.

The first postulate has several consequences which can be tested. In the sequence of reactions leading from acetate to cholesterol, it has been shown that the most sensitive rate-limiting steps lie between acetate and mevalonic acid.⁽⁶⁾ Therefore, if the tracer process were begun with mevalonic acid, or even cholesterol, the differences in synthesis rates might be expected to diminish, if not disappear completely. We have already shown⁽³⁾ that dietary cholesterol appears at different rates in the various liver cholesterol esters; subsequent experiments⁽⁷⁾ with mevalonic acid further confirmed that the esterified sterols are of differing activities from this point in their synthetic pathway.

The second postulate leans on the assumption that the enzymes concerned with the release, transport and activation of fatty acids can distinguish between individual fatty acids. Until lately, such a concept stood alone, unsubstantiated by any independent information. Recently an article by Collins⁽⁸⁾ gave promise of support to such an idea. Collins injected P^{32} into a rat and isolated the liver lecithins. When this fraction was subjected to countercurrent fractionation, a spectrum of lecithins differing in fatty acid composition was obtained. The specific activity of the phosphorus in this spectrum was not uniform but heterogeneous, and he is of the opinion that this can be explained only on the basis of fatty acid specificity in the synthetic processes. The similarity in the phenomena of heterogeneity of phosphorus in lecithins and of cholesterol in sterol esters becomes greater if we consider the phosphorus activity represents the replacement rate of the glycerolphosphorylcholine moiety; in both cases we have an alcohol which is common to a variety of fatty acid esters, and in both cases, the replacement rate of the alcohol in the ester is a function of the fatty acid.

If we now center our attention on the free fatty acid pools, it is clear that the specific activity of the sterol in the ester becomes a secondary reflection of the kinetics of fatty acid metabolism. This shift of emphasis may provide the necessary basis of explanation for some of the

phenomena described above; for example, the "stress" response in labeling pattern might be related to the mobilization of fatty acid by adrenalin, a stress reaction known to be extremely rapid.

This lability of esterification rates has a second significance pertaining to future investigations; such studies will quickly outrun the capabilities of the fractionation procedures. Useful as our methods have been and continue to be, they are clearly not suited to the large numbers of samples which will be generated by detailed examination of these phenomena; nor in all likelihood will it be possible to extend their range downward to permit multiple observations on the same animal. The wide limits within which esterification can vary, coupled with the variety of physiological conditions in which the animal may exist, render the problem unmanageable when small numbers of animals are dictated by methodology. Further investigation by these means must, therefore, await another breakthrough which simplifies or extends the technique without sacrificing its inherently useful features.

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CALCULATION OF ΔF° OF DEUTERO-UREA

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Urea plays critical roles in the nitrogen metabolism of virtually all living organisms and is of considerable interest as an organic compound. Thomson and Klipfel⁽¹⁾ showed that the quantity of urea in the blood of nephrectomized rats accumulated 25% faster when the rats were given heavy water rather than normal water. Ruehrwein and Huffman⁽²⁾ and Huffman⁽³⁾ measured the heat capacity and heat of combustion of urea and, hence, obtained its standard free energy of formation. By means of this quantity and known free energies of formation for CO_2 (g), H_2O (g) and NH_3 (g), they obtained a value of 254 cal/mole at 298.16°K for the standard free energy for the reaction CO_2 (g) + 2 NH_3 (g) $\rightarrow$ $\text{CO}(\text{NH}_2)_2$ (s) + H_2O (g).

When hydrogen atoms in urea, ammonia, and water are replaced by deuterium atoms, the force fields of the respective molecules are unchanged although new sets of frequencies result. Therefore, the free energy change of the equilibrium between deuterio-ammonia and carbon dioxide to yield deuterio-urea and heavy water can be calculated by means of known spectroscopic data. Even though it is not possible to make a careful comparison of our calculation with that of the thermochemical value because of insufficient calorimetric data for deuterio-urea, at least it is of value to examine whether the formation of the deuterium compound is favored over that of the hydrogen compound in biological processes.

In this report, we have calculated ΔF_f° , ΔH_f° , ΔS° , $F^\circ - H_0^\circ/T$, $H^\circ - H_0^\circ/T$, C_p of deuterio-urea and urea from spectroscopic data⁽⁴⁻⁸⁾ and ΔF_f° of deuterio-ammonia from the equilibrium constant of partition functions.^(9,10) These data have been used in conjunction with other calorimetric data^(2,3) to obtain the standard free energy change in the reaction:

The Raman⁽¹¹⁾ and infrared⁽⁴⁻⁸⁾ spectra of deuterio-urea have been reported in the literature, but no consistent assignment of the vibrational frequencies has been given. With the recently published assignment for a structurally similar molecule, with normal urea^(4,5,8) as a basis, it is possible to make a reasonable choice for the fundamental vibrational frequencies of deuterio-urea. This choice of frequencies, together with an approximate description of vibrational modes, is given in Tables 23 and 24.

Using the frequencies in a harmonic oscillator model⁽¹²⁾ and assuming the structural parameters⁽⁴⁾ given in Figure 40, one can calculate the approximate thermodynamic functions for urea at one atmosphere. Values of these functions are given in Table 25.

TABLE 23

Fundamental vibration frequencies for urea, (NH₂)₂CO*

Using the frequency assignment given, and the Table of Einstein Functions⁽¹²⁾ the contributions for each fundamental mode are obtained by interpolation and summation. These computations are summarized as follows:

Description, γ_i	Assignment	Reference	Frequency, cm ⁻¹	At 298.16°K $\gamma_i \frac{hc}{RT}$ $x_i = \frac{\gamma_i hc}{RT}$ $= 0.00482 \gamma_i$	$\frac{x}{e^x - 1}$	$\frac{x^2 e^x}{(e^x - 1)^2}$	$-\ln(1 - e^{-x})$
γ_1	asymmetrical N-H, stretch	4	3449	16 6242	-	0 00002	-
γ_2	symmetrical N-H, stretch	4	3362	16 2048	-	0 00002	-
γ_3	NH ₂ bend $\gamma(C=O)$ stretch	4,5	1689	8 1410	0 01919	0 01934	0 00030
γ_4	NH ₂ bend and $\gamma(C=O)$	4,5	1610	7 7602	0 00331	0 02570	0 00045
γ_5	NH ₂ rock	4,5	1160	5 5912	0 02096	0 11751	0 00371
γ_6	$\gamma(CN)$ stretch	4,5	1005	4 8441	0 03857	0 18774	0 00793
γ_7	N-C-N, symmetrical skeletal deformation	4,5	558	2 6896	0 19590	0 56534	0 07030
γ_8	asymmetrical N-H, stretch	4	3436	16 5615	-	0 00002	-
γ_9	symmetrical N-H	4	3342	16 1084	-	0 00003	-
γ_{10}	NH ₂ bend	4,5	1630	7 8566	0 00303	0 02386	0 00037
γ_{11}	$\gamma(CN)$ stretch	4,5	1472	7 0950	0 00591	0 04196	0 00083
γ_{12}	NH ₂ rock	5,5	1054	5 0803	0 03179	0 16253	0 00612
γ_{13}	S(NCO) asymmetrical skeletal vibration	4,5	574	2 7667	0 18583	0 54791	0 06470
γ_{14}	CONN, out of plane skeletal, bend	4,5	790	3 8078	0 08630	0 47729	0 02240
γ_{15}	NH ₂ Wag	5	717	3 4559	0 11228	0 40186	0 03226
γ_{16}	NH ₂ Wag	**	641	3 0896	0 14730	0 47687	0 04656
γ_{17}	NH ₂ torsional vibration	4	410	1 9762	0 31821	0 72950	0 14858
γ_{18}	NH ₂ torsional oscillation	**	520	2 5064	0 22279	0 60750	0 08476
$\sum_{i=1}^{18}$			27419		1 39137	4 38500	0 48927

*From the above summations, it is seen that the vibrational contributions at 298.16°K are,

$$\left[\frac{(H^0 - H_0^0)}{T} \right]_{\text{vib}} = R \sum_i \left[\frac{x_i}{(e^{x_i} - 1)} \right] = 1.9872 (1.39137) = 2.76498 \text{ cal/(deg)(mole)} \quad (1)$$

$$- \left[\frac{(F^0 - H_0^0)}{T} \right]_{\text{vib}} = -R \sum_i \left[\ln(1 - e^{-x_i}) \right] = -1.9872 (0.48927) = -0.97228 \text{ cal/(deg)(mole)} \quad (2)$$

$$(C_0^0)_{\text{vib}} = R \sum_i \left[\frac{2 x_i e^{x_i}}{(e^{x_i} - 1)^2} \right] = 1.9872 (4.38500) = 8.7139 \text{ cal/(deg)(mole)} \quad (3)$$

$$S_0^0 = \left[\frac{H^0 - H_0^0}{T} - \frac{F^0 - H_0^0}{T} \right] = 2.7498 + 0.97228 = 3.73726 \text{ eu} \quad (4)$$

$$\omega_0 = \frac{1}{2} \sum_i \gamma_i = 13709.5 \text{ cm}^{-1}$$

$$\omega_0 = \frac{E_0}{hc} = \frac{1}{2} \sum_i \omega_i$$

$$E_0 = \omega_0 hc = 2.86 \times 10^{-4} \omega_0 = 3.921 \text{ kcal/mole} \quad (5)$$

**Estimated

TABLE 24

Fundamental vibration frequencies for deutero-urea, (ND₂)₂ CO*

Description γ_i	Frequency, cm^{-1}	$x_i = \frac{\gamma_i hc}{RT}$ $= 0.00482 \gamma_i$	$\frac{x}{(e^x - 1)}$	$\frac{x^2 e^x}{(e^x - 1)^2}$	$-\ln(1 - e^{-x})$
γ_1	2589	12.479	0.00005	0.00061	0.000004
γ_2	2428	11.703	0.00010	0.00113	0.000008
γ_3	1632	7.866	0.00302	0.02368	0.000382
γ_4	1248	6.015	0.01478	0.08908	0.002457
γ_5	1002	4.830	0.03888	0.18933	0.008019
γ_6	891	4.294	0.05961	0.25928	0.013800
γ_7	476	2.294	0.25716	0.65631	0.106207
γ_8	2586	12.464	0.00005	0.00061	0.000004
γ_9	2433	11.727	0.00009	0.00113	0.000008
γ_{10}	1501	7.234	0.00524	0.03792	0.000725
γ_{11}	1160	5.591	0.02095	0.11758	0.003742
γ_{12}	854	4.116	0.06803	0.28492	0.016378
γ_{13}	512	2.468	0.22901	0.61697	0.088841
γ_{14}	779	3.755	0.08963	0.34505	0.023559
γ_{15}	474	2.285	0.25890	0.65862	0.107334
γ_{16}	473**	2.280	0.25977	0.65978	0.107902
γ_{17}	293	1.412	0.45543	0.84958	0.279903
γ_{18}	371**	1.788	0.35991	0.77197	0.183679
$\sum_{i=1}^{18}$	21702		2.12060	5.56355	0.942952

*An approximate description of the vibrational modes for deutero-urea is same as urea in Table 23.

$$[(H^\circ - H_0^\circ)/T]_{\text{vib}} = 4.2136 \text{ cal}/(\text{deg})(\text{mole})$$

$$-[(F^\circ - H_0^\circ)/T]_{\text{vib}} = 1.8736 \text{ cal}/(\text{deg})(\text{mole})$$

$$[C_p^\circ]_{\text{vib}} = 11.0548 \text{ cal}/(\text{deg})(\text{mole})$$

$$S^\circ = 6.0872 \text{ eu}$$

$$E_0 = 3.103 \text{ kcal/mole}$$

**Estimated

$$\begin{aligned} D(C - O) &= 1.24 \text{ \AA} \\ D(C - N) &= 1.33 \text{ \AA} \\ D(N - H) &= D(N - D) = 1.02 \text{ \AA} \\ \phi &= 110^\circ \\ \alpha &= 109^\circ 28' \end{aligned}$$

Figure 40

Structural parameters of deutero-urea.

TABLE 25

Thermodynamic functions for normal urea $\text{CO}(\text{NH}_2)_2$ and deuterio-urea $(\text{ND}_2)_2$

Substance	T, °K	C_p , cal/deg	$(H^\circ - H_0^\circ)/T$, cal/deg mole	$-(F^\circ - H_0^\circ)/T$, cal/deg mole	S° , eu	$E_1 = hc \gamma_i$, kcal/mole
normal urea $\text{CO}(\text{NH}_2)_2$	298.16	8.7139	2.70	-1/522	4.22	3.921
deuterio-urea $\text{CO}(\text{ND}_2)_2$	298.16	11.0548	4.20	-1/75	5.95	3.103

For $\text{CO}(\text{ND}_2)_2$, since no experimental determinations of ΔH_f° , ΔF_f° or S° are available by calorimetric measurement, an estimate of thermodynamic properties may be secured from consideration of the difference in the zero-point energies of normal and deuterio-urea. On the assumption that bond energies in H_2 and D_2 are the same for isotopic molecules⁽¹⁰⁾ and that bond energies in $\text{CO}(\text{NH}_2)_2$ and $\text{CO}(\text{ND}_2)_2$ are also the same, the difference in zero point energies, $hc \sum_i (\omega_{0,D} - \omega_{0,H})$, between $\text{CO}(\text{ND}_2)_2$ and $\text{CO}(\text{NH}_2)_2$ was calculated to be 818 cal/mole from Table 25. The heats of formation of $\text{CO}(\text{NH}_2)_2$ and $\text{CO}(\text{ND}_2)_2$ at 298.16°K are obtained by considering the chemical equations,

From the heat content functions of $\text{CO}(\text{NH}_2)_2$ and $\text{CO}(\text{ND}_2)_2$, the values of $H^\circ - H_0^\circ$, the heat content at 298.16°K referred to the absolute zero, are obtained by simple multiplications of the heat content function by 298.16°K, viz.,

$$h^\circ[\text{CO}(\text{NH}_2)_2] = H^\circ - H_0^\circ = T \left(\frac{H^\circ - H_0^\circ}{T} \right) = 824.43 \text{ cal/mole}$$

$$h^\circ[\text{CO}(\text{ND}_2)_2] = H^\circ - H_0^\circ = T \left(\frac{H^\circ - H_0^\circ}{T} \right) = 1252.27 \text{ cal/mole}$$

$$h^\circ(\text{H}_2)^{(14)} = H^\circ - H_0^\circ = T \left(\frac{H^\circ - H_0^\circ}{T} \right) = 2023.91 \text{ cal/mole}$$

$$h^\circ(\text{D}_2)^{(13,14)} = H^\circ - H_0^\circ = T \left(\frac{H^\circ - H_0^\circ}{T} \right) = 1967.26 \text{ cal/mole}$$

The quantity $\Delta H_{298.16^\circ\text{K}}^\circ [\text{CO}(\text{NH}_2)_2] = -79,634 \text{ cal/mole}$ was obtained from the literature.⁽²⁾

However, the value of $\Delta H_{298.16}^\circ$ of $\text{CO}(\text{ND}_2)_2$ is obtained from the relationship

$$\begin{aligned}\Delta H_{298.16}^\circ [\text{CO}(\text{ND}_2)_2] &= \Delta H_{298.16}^\circ [\text{CO}(\text{NH}_2)_2] \\ &+ \left\{ \Delta H_0^\circ [\text{CO}(\text{ND}_2)_2] - \Delta H_0^\circ [\text{CO}(\text{NH}_2)_2] \right\} \\ &+ \left\{ h^\circ [\text{CO}(\text{ND}_2)_2] - h^\circ [\text{CO}(\text{NH}_2)_2] \right\} \\ &- 2 \left\{ h^\circ(\text{H}_2) - h^\circ(\text{D}_2) \right\} \quad . \quad (7)\end{aligned}$$

Thus, the quantity, $\Delta H_{298.16}^\circ [\text{CO}(\text{ND}_2)_2] = -79,907 \text{ cal/mole}$.

Because of the structural similarity of the compounds involved, the absolute entropies of $\text{CO}(\text{NH}_2)_2$ and $\text{CO}(\text{ND}_2)_2$ in the solid or liquid state should be almost equal. It is usually possible to make a workable estimate of the entropy of a given compound when the entropy of a closely related compound is known. The quantity, $S_{298.16}^\circ = 25 \text{ eu}$ for $\text{CO}(\text{NH}_2)_2$ was measured calorimetrically by Ruehrwein and Huffman.⁽²⁾ The absolute entropy of $\text{CO}(\text{ND}_2)_2(\text{s})$ was then estimated in the following manner. This estimation assumes that the only difference between the total entropies in the two cases is attributable to the internal vibrations of the molecules, and hence one writes the expression,

$$\begin{aligned}S_{\text{Total}}^\circ [\text{CO}(\text{ND}_2)_2] - S_{\text{vib}}^\circ [\text{CO}(\text{ND}_2)_2] &\cong S_{\text{Total}}^\circ [\text{CO}(\text{NH}_2)_2] \\ &- S_{\text{vib}}^\circ [\text{CO}(\text{NH}_2)_2] \quad (8)\end{aligned}$$

$$\begin{aligned}S_{\text{Total}}^\circ [\text{CO}(\text{ND}_2)_2] &\cong S_{\text{Total}}^\circ [\text{CO}(\text{NH}_2)_2] - S_{\text{vib}}^\circ [\text{CO}(\text{NH}_2)_2] \\ &+ S_{\text{vib}}^\circ [\text{CO}(\text{ND}_2)_2]\end{aligned}$$

$$S_{\text{Total}}^\circ [\text{CO}(\text{ND}_2)_2] \cong 26.73 \text{ eu}$$

This value together with the entropy values 1.361, 34.602, 49.003 and 45.767 eu for graphitic carbon, deuterium, oxygen and nitrogen, respectively,⁽¹³⁾ yield the standard entropy of formation of $\text{CO}(\text{ND}_2)_2$, i.e.,

$$\begin{aligned}\Delta S_{298.16}^\circ [\text{CO}(\text{NH}_2)_2] &\cong S^\circ [\text{CO}(\text{ND}_2)_2] - S^\circ(\text{N}_2) - 2S^\circ(\text{D}_2) - S^\circ[\text{C}] - \frac{1}{2}S^\circ(\text{O}_2) \\ \Delta S_{298.16}^\circ &\cong -114.103 \text{ eu} \quad (9)\end{aligned}$$

After obtaining the values of $\Delta H_{298.16}^\circ$ and $\Delta S_{298.16}^\circ$ for deutero-urea from these calculations, one can calculate its standard free energy of formation by substitution into the equation

$$\Delta F_{298.16}^\circ = \Delta H_{298.16}^\circ - T\Delta S_{298.16}^\circ = -45,886 \text{ cal/mole} \quad . \quad (10)$$

The value of the equilibrium constant, K_D , for ND_3 is derived from a consideration of the following chemical equilibria:

$NH_3 + NHD_2 = 2NH_2D$	K_6
$NHD_2 + D_2 = ND_3 + HD$	K_9
$NH_3 + ND_3 + HD = D_2 + 2NH_2D$	K_6/K_9
$2NH_3 + 2HDO = 2NH_2D + 2H_2O$	$(K_{10})^2$
$ND_3 + HD + 2H_2O = D_2 + NH_3 + 2HDO$	$K_6/K_9(K_{10})^2$
$2H_2O + 2HD = 2HDO + 2H_2$	$(K_1)^2$
$ND_3 + 2H_2 = 2H_2 = D_2 + NH_3 + HD$	$\frac{K_6}{(K_{10})^2 K_9 (K_1)^2}$
$\frac{1}{2} H_2 + \frac{1}{2} D_2 = HD$	$(K_2)^{1/2}$
$ND_3 + \frac{3}{2} H_2 = \frac{3}{2} D_2 + NH_3$	$\frac{K_6}{(K_{10})^2 K_9 (K_1)^2 (K_2)^{1/2}}$
$NH_3 = \frac{1}{2} N_2 + \frac{3}{2} H_2$	K_H
$ND_3 = \frac{1}{2} N_2 + \frac{3}{2} D_2$	K_D
$\frac{1}{K_D} = \frac{K_6}{K_{10}^2 K_9 K_1^2 K_2^{1/2} K_H}$	
$K_D = \frac{K_{10}^2 K_9 K_1^2 K_2^{1/2} K_H}{K_6}$	(11)

Kirshenbaum⁽¹⁰⁾ and Kimball and Stockmayer⁽⁹⁾ calculated the equilibrium constants (K_1 , K_2 , K_6 , K_9 , K_{10}) by evaluating the ratios of partition functions as a function of temperature for isotopic species. In order to obtain the thermodynamic equilibrium constants, the partition function equilibrium constants are plotted against $1/T$ on semilogarithmic paper and the resulting straight lines are extrapolated to the respective intercepts. These intercepts give the respective thermodynamic equilibrium constants.

This line of reasoning is substantiated in the following manner. According to a theorem of thermodynamics the equilibrium constant, K , can be expressed in terms of the free energies F° of the gases involved as follows:

$$\Delta F^\circ = \Delta H_0^\circ + \sum_i F_i^\circ \text{ products} - \sum_i F_i^\circ \text{ reactants} = -RT \ln K \quad , \quad (12)$$

in which ΔH_0° is the standard zero-point energy change, F_i° is the free energy function multiplied by T , i.e., $T(F^\circ - H_0^\circ)/T$, and is defined as $F_i^\circ = -RT \ln Q_i$. Substitution of this quantity in Eq. (12) yields the equation,

$$-RT \ln K = \Delta H_0^\circ - RT \sum_i \ln Q_i \text{ products} + RT \sum_i \ln Q_i \text{ reactants} \quad (13)$$

or

$$-RT \ln K = \Delta H_0^\circ - RT \ln \frac{\prod_i Q_i \text{ products}}{\prod_i Q_i \text{ reactants}} \quad , \quad (14)$$

In view of Eq. (12), Eq. (14) can be written as

$$-\ln K = \frac{\Delta H_0^\circ}{RT} - \ln \left[\frac{\prod_i Q_{ip}}{\prod_i Q_{in}} \right] \quad . \quad (15)$$

Defining $\xi = \frac{\prod_i Q_p}{\prod_i Q_r}$, ratio of products of partition functions, one writes that

$$\ln \xi = \Delta H_0^\circ / RT + \ln K \quad , \quad (16)$$

which equation yields a straight line when plotted as $\ln \xi$ vs $1/T$.

The results of this treatment are shown in Figure 41.

Figure 41

Graph for extrapolating the thermodynamic equilibrium constants (K_6 , K_9 , K_8 , K_{10} , K_1 , K_2) for deuterium exchange reactions involving hydrogen, water and ammonia.

From a knowledge of ΔF° for the process, $\text{NH}_3 \rightarrow \frac{1}{2} \text{N}_2 + \frac{3}{2} \text{H}_2$, the thermodynamic equilibrium constant, K_H of $\text{NH}_3(\text{g})$ is calculated by means of the thermodynamic relationship,

$$\Delta F_{298.16}^\circ = -RT \ln K_H \quad ,$$

in which $\Delta F_{298.16}^\circ$ ⁽¹³⁾ = -3910 cal/mole

Hence,

$$K_H = 695.$$

The equilibrium constant K_D of $\text{ND}_3(\text{g})$ is obtained by substitution into Eq. (11) which yields the result,

$$K_D = \frac{(695)(0.48)(1.5)^2(0.7)^2(4.2)^{1/2}}{3} = 242 \quad . \quad (18)$$

The standard free energy of formation, $\Delta F_{298.16}^\circ$ of ND_3 is calculated directly from the expression,

$$\Delta F^\circ = -RT \ln K_D$$

in which $K_D = 243$.

Hence, $\Delta F^\circ = -3270 \text{ cal/mole}$.

In view of our calculated free energy of formation for deuterio-urea and deuterio-ammonia, and also of the availability of free energy of formation for D_2O and carbon dioxide, it is interesting to calculate the free energy of formation of the deuterio-urea from equilibrium data (see Table 26).

TABLE 26

Equilibrium data used to calculate the free energy of formation of deuterio-urea

Substances	$\Delta H_{298.16}^\circ$, cal/mole	$\Delta S_{298.16}^\circ$, cal/deg	$\Delta F_{298.16}^\circ$, cal/mole	Reference
deutero-urea $\text{CO}(\text{ND}_2)_2$	-79,907	-114.103	-45,886	2,3
urea $\text{CO}(\text{NH}_2)_2$	-79,634	-109.056	-47,118	
$\text{ND}_3(\text{g})$			- 3,270	
$\text{NH}_3(\text{g})$	- 1,104	46.01	- 3,976	13
$\text{D}_2\text{O}(\text{g})$	-59,562	47.379	-56,067	13
$\text{H}_2\text{O}(\text{g})$	-57,797	45.106	-54,635	13
$\text{D}_2(\text{g})$		34.602		14,15
$\text{H}_2(\text{g})$		31.211		14,15

The calculation from the equilibrium data may be summed up as follows:

The values of the standard free energies of formation and other necessary data are tabulated in Table 26.

From the equilibrium data, Ruehrwein and Huffman⁽²⁾ find for the free energy of reaction, $\text{CO}_2(\text{g}) + 2\text{NH}_3(\text{g}) = \text{CO(NH}_2)_2(\text{s}) + \text{H}_2\text{O(g)}$ at 298.16°K, 254 calories. The free energy for the corresponding reaction involving deuterated compounds is more negative by 1,699 calories.

It is interesting to observe, in view of these free energies, that if one considers the two systems (hydrogen and deuterium) separately, the equilibrium in the case of the deuterium system is shifted to a larger equilibrium concentration of the urea compound. The experiment of Thomson and Klipfel⁽¹⁾ demonstrated that administration of D_2O to rats increased urea. Although the reason for this increase is not entirely clear, the equilibria considerations cited above would favor an increase in urea levels. However, the actual system involved is more complex in that exchange reactions occur, and one must then consider reactions involving exchanges to see if such a system predicts an increase in total urea.

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EVIDENCE FOR THE PARTICIPATION OF METALS IN A TRANSMETHYLATION

Stanley K. Shapiro and David A. Yphantis

Partially purified homocysteine-transmethylases have been studied by various investigators,⁽¹⁻⁴⁾ but participation of metal ions has not been described. This report presents evidence suggesting that the S-methyl-methionine and S-adenosylmethionine-homocysteine transmethylases of various microorganisms are metalloenzymes.

The preparation of the homocysteine transmethylases of *Saccharomyces cerevisiae*, *Aerobacter aerogenes*, *Escherichia coli* K12 and rat liver has been described previously.^(5,6) Enzymatic activity was measured with our tracer assay.⁽⁶⁾ Virtually full transmethylase activity is retained after dialysis of preparation of *S. cerevisiae*, *A. aerogenes*, and *E. coli* K12 against several changes of 100 volumes of 0.01 M phosphate buffer, pH 6.5, for 48 hours at 1°C. However, dialysis for 16 hr against 10^{-3} M ethylenediaminetetraacetic acid (EDTA), 10^{-3} M 1, 10-orthophenanthroline (OP) or 10^{-3} M 8-hydroxyquinoline (8-HQ) reduces the activities by a factor of at least five. Activity is also inhibited to the same extent a few minutes after direct addition of these chelating agents. One-half of the transmethylase activity remained after addition of EDTA to a concentration of $\sim 3 \times 10^{-6}$ M, OP to a concentration of $\sim 3 \times 10^{-5}$ M or 8-HQ to a concentration of $\sim 3 \times 10^{-4}$ M. The maximum inhibitions observed were 80% with EDTA at levels greater than 5×10^{-5} M, 97% with OP at levels greater than 10^{-3} M and 90% with 8-HQ at a concentration of 10^{-2} M, the highest concentration used. The lack of complete inhibition by EDTA at the higher concentrations (Figure 42) suggests a heterogeneity in the configuration or composition of the enzyme.

Figure 42

Enzyme activity as a function of concentration of EDTA. Each reaction mixture contained per ml: $8 \mu\text{M}$ L-homocysteine, $8 \mu\text{M}$ S-methyl-L-methionine (C^{14}H_3), 0.1 M phosphate buffer, pH 6.5 and sufficient yeast enzyme to produce 0.2 to $0.3 \mu\text{M}$ of methionine per ml of reaction mixture in one hour at 35°C .

The inhibited enzymes have been reactivated by (1) addition of certain metal ions, (2) dilution of the inhibited enzyme or (3) dialysis against phosphate buffer. Table 27 shows the reactivation by metal ions of yeast S-methylmethionine-homocysteine transmethylase inhibited by EDTA and OP. The apparent order of reactivation was: $\text{Zn}^{++} > \text{Cd}^{++} > \text{Ni}^{++} > \text{Co}^{++} > \text{Mn}^{++} > \text{Cr}^{++} > \text{Fe}^{++}$ and Fe^{+++} . The ability of metal ions to reactivate the inhibited enzyme is in general agreement with their relative binding capacities for the inhibitors. One would expect Cu^{++} to reactivate; however 10^{-4} M Cu^{++} completely inhibits the original enzyme. Both Fe^{++} and Fe^{+++} ions would be expected to be very active but show only limited reactivation. The other nontoxic metal ions tested, Ca^{++} , Mg^{++} and Sn^{++} , were inactive. Reactivation above 100% probably reflects the fact that Zn^{++} increases the activity of the original preparations by 5 to 10%.

TABLE 27

Metal reactivation of S-methylmethionine-homocysteine transmethylase after inhibition with chelating agents

Addition	Percent of original activity*			
	EDTA, 10^{-4} <u>M</u>		OP, 2×10^{-4} <u>M</u>	
	Metal = 10^{-4} <u>M</u>	Metal = 4×10^{-4} <u>M</u>	Metal = 2×10^{-4} <u>M</u>	Metal = 8×10^{-4} <u>M</u>
Zn^{++}	110	116	103	108
Cd^{++}	87	112	84	92
Ni^{++}	80	105	73	78
Co^{++}	73	78	61	68
Mn^{++}	55	102	55	66
Cr^{++}	42	97	52	56
Fe^{++}	30	54	46	46
Fe^{+++}	32	42	51	54
None	16		17	

*Original activity refers to the enzyme activity obtained with control mixtures in the absence of inhibitor. The reaction mixtures were prepared as described for Figure 42

Preincubation of yeast S-methylmethionine-homocysteine transmethylase with 10^{-4} M EDTA for one hour followed by a 1:5 dilution with 10^{-4} M EDTA in 0.1 M phosphate buffer, pH 6.5, results in a preparation with 24% of the activity of the control mixtures (Table 28). If the 1:5 dilution was made with phosphate buffer alone so that the final EDTA concentration was 2×10^{-5} M, 77% of the control activity was found, compared to 74% for an aliquot of the original preparation maintained at 2×10^{-5} M before and after the 1:5 dilution. Similarly, it was possible to reverse OP inhibition by dilution. These results suggest that the metal is firmly bound to the enzyme and is not removed by the chelating agents.

TABLE 28

Reversal of inhibition of S-methylmethionine-homocysteine transmethylase activity by dilution.

Inhibitor	Original Concentration, <u>M</u>	Concentration after dilution, <u>M</u>	Percent of original activity*
EDTA	10^{-4}	10^{-4}	24
EDTA	10^{-4}	2×10^{-5}	77
EDTA	2×10^{-5}	2×10^{-5}	74
OP	10^{-4}	10^{-4}	22
OP	10^{-4}	2×10^{-5}	59
OP	2×10^{-5}	2×10^{-5}	57

* Original activity refers to the enzyme activity obtained with control mixtures in the absence of inhibitor. The reaction mixtures were prepared as described for Figure 42.

The homocysteine transmethylases of A. aerogenes, E. coli K12 and S. cerevisiae with either S-methylmethionine or S-adenosylmethionine as methyl donors, are inhibited by these chelating agents and may be similarly reactivated. On the other hand, the S-methylmethionine and S-adenosylmethionine-homocysteine-transmethylases of rat liver ⁽⁶⁾ are not inhibited by dialysis against 10^{-3} M EDTA or 10^{-3} M OP. Thetin-homocysteine transmethylase activity of liver extract has been reported to be unaffected by EDTA. ^(1,2) The observations reported here suggest a significant difference between microbial and mammalian homocysteine transmethylases. The evidence at hand suggests that these microbial homocysteine transmethylases are metalloenzymes as defined by Vallee. ⁽⁷⁾ There is a strong metal-enzyme bond since these enzymes are stable to repeated dialysis and the inhibition caused by chelating agents is reversed by dilution or dialysis. Reactivation by metal ions probably consists of titration of the chelating agent bound to the enzyme. However, identification of the metal involved and definitive evidence that these are metalloenzymes rather than metal-enzyme complexes will only be possible with highly purified enzyme preparations.

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SOME EFFECTS OF ULTRAVIOLET IRRADIATION ON YEAST CELLS (CANDIDA UTILIS).

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Ultraviolet irradiation of cells results in injury which, in the case of yeast cells, is manifested by mutation, interference with budding, or cell death, in the order of increasing dose. A majority of the studies of the past have dealt with genetic effects and reduction in viability. Nadson and Stern,⁽¹⁾ whose work has been overlooked by many investigators, gave an account of the morphological changes occurring in irradiated yeast cells as observed with visible light microscope optics. Injury resulting in the release of organic compounds into the surrounding medium has been studied, especially by Loofbourov and his co-workers,^(2,3) while more recent investigations have emphasized the permeability changes of the cell membrane toward inorganic ions.^(4,5,6)

The paucity of data on the morphological and cytological changes produced in yeast cells by ultraviolet irradiation suggested a detailed study. Furthermore, it was desired to clarify details of the mechanism of release of ultraviolet-absorbing organic cell constituents into the medium after ultraviolet irradiation. In particular, information was sought concerning the radiation dose necessary for the release, the quantity and type of compounds concerned, and the location or cellular compartments from which these substances are released. The study was facilitated by the availability of Candida utilis in two distinct conditions as a consequence of nutritional regimen; these cells differ widely in their absorption of ultraviolet energy.⁽⁷⁾ No less important was the availability of newly developed ultraviolet microscope optics and instrumentation.

Materials and Methods

Candida utilis, strain ATCC 9950, was used. Cells grown on a minimal culture medium containing salts, NH_4^+ , and glucose have a low ultraviolet absorption and were used as control cells. The presence of sulfur amino acids in the culture medium, notably L-methionine, L-homocysteine, S-methyl-L-methionine, each in a concentration of $5 \mu\text{M/ml}$, causes the formation of S-adenosylmethionine, which is stored in the vacuole.⁽⁷⁾ Because of its high concentration and the intense absorption of the adenine moiety at $260 \text{ m}\mu$, the cells grown on this culture medium show a much higher ultraviolet absorption than control cells. For convenience, the cells containing S-adenosylmethionine are referred to as "AM cells," in contrast to the "control cells" described above. All experiments were carried out with cells from cultures that had been incubated for 40 to 44 hours at 30°C ; budding had come to an end at this time. Other details of culture have been

described before,⁽⁸⁾ except that there was a five-fold increase of K_2HPO_4 and KH_2PO_4 in the culture medium. This measure resulted in greatly increased yields of S-adenosylmethionine. Water was used for washing and suspending the cells in the experimental procedures. The techniques of extraction, ion exchange chromatography, and spectrophotometry have been reported earlier.⁽⁹⁾

Ultraviolet energy was obtained from a "Mineralight," model SL 2537, and was measured with a Latarjet meter.⁽¹⁰⁾ Most of the energy fell in the 253.7 m μ region, only 3% of the total being above 300 m μ . Five-milliliter quantities of 1% suspension of cells in distilled water were irradiated from above in Petri dishes 6 cm in diameter, a 1-mm Vycor plate replacing the standard cover. A correction was made for the absorbance of this material. Unless specified otherwise, the dose rate was 35 erg/mm²/sec. Agitation during irradiation was accomplished by means of a motor-driven rocking table tilting 5° above and 5° below horizontal once each second. The temperature during irradiation was 25°C. Low levels of room illumination were maintained to limit photoreactivation. Viability was determined from colony counts obtained from quadruplicate platings on potato dextrose agar.

Descriptions of the ultraviolet microscope and methods of using it have been reported previously.^(7,11) A set of new Carl Zeiss Ultrafluor optics has been added. These consist of a 32 x / 0.40 NA and a 100 x / 0.85 NA glycerine immersion objective, an achromatic UV condenser / 0.8 NA, with iris diaphragm and a UV projective 10:1. Observations were also made with an A. O. Baker interference microscope using the 100 x shearing system.

Results

The differences in the composition of Candida utilis obtained from culture medium with and without sulfur amino acid supplement are presented in Table 29. S-Adenosylmethionine accounts for more than half of the total optical density of the perchloric acid extracts of the enriched cells; these extracts contain the total of nucleic acids, nucleotides, and related compounds. Under the present conditions of acid hydrolysis and chromatography with cation exchange resin,⁽⁷⁾ the pyrimidine compounds appear in the initial fraction of the acid eluate. Their individual determination has not been attempted; however, the total optical density of this fraction has been listed in Table 29 to illustrate that the organic sulfur supplement does not exert a major influence on their quantity; the same may be seen for guanine. The difference in the adenine values is accounted for by the presence of S-adenosylmethionine in the cells obtained from supplemented culture medium. On a dry weight basis, the total nucleic acid content of both types of cells is about 4%; that of S-adenosylmethionine in AM cells, calculated as

the sulfonium ion (MW 399), in the experiments I b and II b of Table 29 is 6.5 and 4.9%, respectively. AM cells offer several advantages over control cells because the large quantity of ultraviolet-absorbing material facilitates observation of the internal organization of the cell by ultraviolet photomicrography. The localization of the adenine sulfonium compound in the vacuole⁽⁷⁾ makes it possible to gain information about the influence of ultraviolet irradiation on the permeability of the vacuolar membrane as well as of the cell membrane.

TABLE 29

Ultraviolet-absorbing compounds in *Candida utilis*

Supplement in the culture medium	Yield of cells, moist weight, g/100 ml of medium	Products of acid hydrolysis/g yeast			
		S-Adenosyl-methionine, μ moles/g	Pyrimidine nucleotide fraction, Optical density*	Guanine, μ moles	Adenine, μ moles
I a None	3.5	1.9	124	9.3	9.2
I b L-Methionine, L-homocysteine, S-methyl-L-methionine (5.0 μ M of each per ml)	1.9	33.3	130	8.7	41.3
II a None	3.4	1.3	146	7.9	10.7
II b Same as in I b	2.0	24.4	152	9.6	33.8

* O.D. units (260 $m\mu$) x ml of hydrolyzate.

The culture medium contained salts, NH_4^+ , and glucose. The time of culture was 44 hours at 30°. All data pertain to moist yeast centrifugate of $80 \pm 3\%$ water content.

Experiments using S-adenosylmethionine as an optical tracer substance depend on the stability of this compound toward ultraviolet irradiation. This was tested by exposing a highly purified preparation⁽⁹⁾ in water solution to 2.9×10^5 erg/mm², in a concentration of 1.7 μ M/ml in a 2-mm layer, and 0.05 μ M/ml in a 5-mm layer. In neither case was any significant change of the absorption spectrum noted, and ion exchange chromatography⁽⁹⁾ of the more concentrated solution ascertained that the sulfonium group had remained intact.

Morphological Changes Occurring in the Yeast Cell after UV Irradiation. During our earlier photomicrographic studies of yeast,⁽⁷⁾ it became evident that changes in the cells are produced by the ultraviolet

energy. With energy levels necessary to obtain photographic images it was usually possible to obtain at least one set of photographs of a field that did not show radiation effects; sometimes the same field could be photographed two or three times with no obvious change. Occasionally, many photographs could be made before changes appeared.

To record these effects, cells that had not been previously irradiated were photographed at 1-min intervals with UV of 265-m μ wavelength, the total dose being increased each time by the amount required for the exposure. While our UV microscope is equipped with devices to monitor the intensity of the UV and to produce integrated, automatically-controlled exposures, we do not have facilities for measuring the dose in the specimen plane. We can only resort to comparing the biological effects with those obtained in our other experiments where the dose has been measured. Results are illustrated in Figure 43.

Figure 43. *Candida utilis*. Ultraviolet (265 m μ) photomicrographs of control and AM cells showing the effects of ultraviolet (265 m μ) irradiation. Sequential pictures obtained at 0, 2, and 4 minutes in each case. (a) normal cell, (b) enlarged vacuole, (c) collapsed cell.

The first change is a sudden increase in the size of the vacuole with increased absorption of UV by the condensed cytoplasm (Figure 43 b). Following this, there is a gradual decrease in cell size, until, again quite suddenly, the volume of the vacuole and cell decreases considerably with a concomitant precipitation of the cytoplasm (Figure 43 c).

With the control cell illustrated, the normal cell appearance (Figure 43 a) was recorded at the 0- and 1-min intervals, the enlarged vacuole (Figure 43 b) at the 2- and 3-min intervals, and the collapsed cell (Figure 43 c) at the 4- through 17-min interval, at which time the series was ended. Another cell in the same field exhibited a normal appearance at 0, the appearance of b at 1 minute, and that of c at 2 minutes. Still another appeared as a at intervals 0, 1, 2, 3 and 4 minutes; as b, at 5 and 6 minutes; and as c at 9 through 17 minutes. In the case of the second cell it was possible to determine that the change from b to c took place in less than 6 sec. General observations suggest that the velocity of the change is linked to the age or metabolic state of the cell, older cells reaching the c state rapidly. AM cells respond in much the same way, but under certain conditions the centrosome is drawn into the vacuole during its violent enlargement to give the appearance of a large dancing body.

The changes occurring during irradiation have also been followed by phase microscopy by examining small samples taken at intervals from suspensions of AM cells and control cells irradiated for 150 minutes at a rate of 35 erg/sec/mm². The 10-min sample showed normal AM cells and control cells of types a and b. At 20 minutes and 40 minutes, both AM and control samples contained a and b types. At 60, 90, and 120 minutes, the AM samples contained b and c type cells, while at 60 minutes and thereafter only c types were observed in the control. At 230 minutes (80 minutes after completion of the irradiation) there were still a few b cells seen in the AM sample, but the rest were of type c in both AM and control samples.

A mixture of control and AM cells mounted on the same slide permitted observation of the response of the two types of cells exposed to the same source of radiation under identical conditions (Figure 44). Here, one may observe the morphological changes and differences in the speed of response of the various cells that we have noted. These cells may be compared to those in Figure 45, where the dose is known. Few cells of type b (Figure 43) can be observed since they exist in that form for but a short time. The cells of type c show less ultraviolet absorption than in the previous figures because of leaching which has occurred in the longer period before the photographs were made.

Release of Ultraviolet Absorbing Material from the Cells. Earlier observations of Loofbourow⁽³⁾ on the release of nucleotides and nucleic acid fragments from yeast cells after ultraviolet irradiation made it desirable to correlate this process with the morphological changes recorded in Figures 43 to 45. The availability of AM cells afforded an opportunity of extending the observations to the vacuolar permeability.

Preliminary experiments were done to determine whether the release of ultraviolet-absorbing constituents preceded, paralleled, or followed the decline in viability of the cells.

Figure 44. A mixture of control and AM cells photographed at 1-min intervals with 265 m μ ultraviolet. The cells received only the amount of radiation used to obtain the pictures.

Figure 45. *Candida utilis*. Ultraviolet (265 m μ) photomicrographs of control cells (left column) and AM cells (right column) irradiated with 253 m μ ultraviolet of 35 erg/sec/mm² for 10, 20, 30, and 60 minutes (top to bottom). The photographic exposures were made after the cells had been allowed to stand for three hours at 25° after irradiation.

Plating dilutions of the irradiated samples showed (see Table 30) that with a 1% suspension of cells in 2-mm layer receiving 35 erg of radiation per mm^2/sec , less than 1% of the control cells retained the ability to form colonies after 4 minutes, while AM cells were somewhat more resistant. Because of the relatively high cell density in the suspending water, the effectiveness of the ultraviolet radiation is suboptimal with respect to killing action. For the present investigation, a suspension containing 1% of cells was chosen to get sufficient material for reliable spectrophotometric analysis of the released ultraviolet-absorbing compounds.

TABLE 30

Fraction of survivors after ultraviolet irradiation of *Candida utilis**

Time of exposure to ultraviolet radiation, min	Survivors (plate count), %	
	Control cells	AM cells
0	100	100
2	37	58
3	7	22
4	0.5	1.4

* The experimental conditions are given in the text.

It was found that the loss of viability precedes the release of ultraviolet absorbing material into the surrounding medium. In all experiments a considerable gap between these two events was noted (Table 30 and Figure 46).

Figure 46

The relation between the dose of ultraviolet irradiation and the quantity of ultraviolet-absorbing material released from cells of *Candida utilis*. Control cells and AM cells (C-1 and AM-1) were kept in water at 5° for 24 hours prior to centrifugation and spectrophotometry of the supernatant solution; cultures C-2 and AM-2 were held for 3 hours at 25° after the ultraviolet exposure. The release in experiments AM-2 and C-2 amounts to 98% and 87% of the total quantity of ultraviolet-absorbing compounds as judged by exhaustive extraction of the cells with 1.5 N perchloric acid. (7)

Despite the small dimensions of the yeast cells and the large surface area in relation to their volume, the transfer of cellular constituents into the surrounding medium after exposure to ultraviolet energy takes some time (see Figure 47). This delay might be explained by a gradual breakdown of the cellular nucleic acids into small, diffusible units, or by a slow, perhaps enzymatic, alteration of the cellular membranes. Samples of AM cells and control cells which had received one hour of UV radiation were kept at 5° for the times specified in Figure 47; after centrifugation at low temperature the optical density at 260 m μ of the supernatant fluid was measured after appropriate dilution. Without previous ultraviolet irradiation neither AM cells nor control cells shed significant amounts of absorbing material. Based on these observations, measurements on all irradiated yeast suspensions were made after 20 to 30 hours of storage at low temperature. An alternative was postirradiation incubation at 25° for three hours; under these conditions the quantity of ultraviolet-absorbing material is higher and approaches the amounts extractable with perchloric acid;⁽⁹⁾ the latter procedure includes the nucleic acids.⁽⁷⁾ The experiments recorded in Figure 46 illustrate the alteration in retentiveness of the cells with increasing quantities of UV irradiation. Below a certain dose, the effect on release is negligible; thereafter, a sudden alteration of the cells occurs, permitting release of ultraviolet-absorbing material both through the vacuolar membrane and the cell membrane. It appears that degradation of the nucleic acids at the higher temperature during the radiation and post-irradiation period (Figure 46, experiments C-2 and AM-2 is responsible for the larger quantities of material released from the cells into the surrounding medium. In both instances, S-adenosylmethionine is released quantitatively as indicated by the optical density of the extracts compared with that of the extracts from control cells. There was no difference in the leaching effect between water and 0.1 M potassium phosphate buffer of pH 7.

Figure 47. The release of ultraviolet-absorbing cellular constituents from *Candida utilis* after ultraviolet irradiation. Experimental details are given in the text.

Ion exchange chromatography⁽⁹⁾ showed that S-adenosylmethionine emerges unimpaired from the vacuole and yeast cell after ultraviolet irradiation; there is no enzymatic decomposition in transit. The quantities found in the suspending water (Table 31) are similar to those extracted from the cells with perchloric acid.⁽⁸⁾ Criteria of identification of S-adenosylmethionine were the characteristic elution from a cation exchange column with strong acid,⁽⁹⁾ the absorption spectrum,⁽⁷⁾ and the extreme sensitivity of the material to alkali, resulting in a shift of the absorption maximum from 260 m μ at neutrality to 268 m μ in alkaline medium.⁽¹²⁾

TABLE 31

Chromatographic analysis of compounds released from yeast cells after UV irradiation

Ultraviolet exposure, min	Analysis of supernatant fluid by Dowex 50 H ⁺ column	
	1 <u>N</u> acid eluate, optical density at 256 m μ /g of cells ^a	6 <u>N</u> acid eluate, optical density at 256 m μ /g of cells ^a
45	217	302
120	242	310
No UV; cells were extracted with 1.5 <u>N</u> perchloric acid	330	315

^a O.D. units x ml of eluate.

A water suspension containing 1% of AM cells was exposed to ultraviolet irradiation as specified in the section on Materials and Methods. After 3 hr at room temperature, the suspension was centrifuged and the supernatant fluid analyzed. All nucleic acid fragments are in the 1 N acid eluate while the 6 N acid fraction contains S-adenosylmethionine.⁽⁹⁾ Extraction with 1.5 N perchloric acid gives a measure of the total material of each type present in the cells.

Discussion

Nadson and Stern⁽¹⁾ using Saccharomyces cerevisiae, have stated that the irradiated cells pass through three phases. In the first of these the vacuoles increase in size, and the cells swell. In the second phase the protoplasm becomes less permeable and more granular, and the fat droplets increase in size and number. Finally, the volume of the vacuole and cell diminishes, and the cell collapses and perishes.

Our observations on Candida utilis vary but slightly from those of Nadson and Stern.⁽¹⁾ Cells exposed to small amounts of ultraviolet irradiation may appear morphologically normal (Figure 43 a) but be incapable of producing colonies. With an increased amount (2×10^4 erg/mm²) a sudden increase in volume of the vacuole occurs. This is followed by a gradual decrease in cell volume for a short time, after which a sudden collapse occurs. The expansion of the vacuole must be interpreted either as an alteration in the physical state of cellular constituents resulting in drastic osmotic changes, or as an effect on membrane permeability.

Correlation of the morphological and biochemical data (Figures 43-46) indicates that normal appearing cells and cells with enlarged vacuoles are present during the period when cellular constituents are being released but slightly. As more material is released, an increased number of cells with enlarged vacuoles as well as collapsed cells may be seen. The rapid rise in rate of release is correlated with the sudden collapse of an increased number of cells with enlarged vacuoles. The amount of material measured in the supernatant fluid is determined by the complete release of material from a limited number of cells, rather than by a limited degree of release from all cells. After the maximum of optical density of the medium has been reached (Figure 47), only collapsed cells may be seen. Nadson and Stern⁽¹⁾ believed that death of the cells occurs at the time of collapse, while our present data (Table 30 and Figure 46) show a wide margin between the dose of ultraviolet causing death and morphological changes respectively. The difference in the doses necessary to bring about the two events does not exclude a relationship between the fundamental mechanisms which are responsible.

The ultraviolet absorbancy displayed by the cells in the photomicrographs (Figures 44 and 45) shows a correlation with the amount of extracted material. AM cells release all S-adenosylmethionine in addition to the compounds found in the suspending fluid of control cells. If the conditions after ultraviolet irradiation are suitable, breakdown of the nucleic acids into diffusible units occurs, and rather clear cell residues are observed (Figure 45).

The wavelengths used in our study for irradiation and for photomicrography were selected for technical reasons. Perhaps they do not provide a maximum effect on the membrane. The data in Table 30 and Figure 45 suggest a somewhat higher resistance of the AM cells. The opposite would be expected if the high absorbance of ultraviolet energy by S-adenosylmethionine could be transmitted to more vital parts of the cell. One may be tempted to interpret this resistance as a shielding effect of the intravacuolar adenine compound. It should be kept in mind, however, that the two types of cells used in this study differ somewhat with respect to nutrition and growth rate (Table 29).

The present data extend Loofbourow's observations on the release of ultraviolet-absorbing material from yeast cells.^(2,3) One of the differences is the use of Candida in our experiments instead of commercial Saccharomyces. In our experience, the former lends itself somewhat better to ultraviolet photomicrographic studies. Our use of a 1% suspension offers some advantages over the 10% yeast suspensions used by Loofbourow⁽²⁾ because the radiation time of 8 hr used in the latter case obscures the sequence of events. In exploratory experiments we have observed that Saccharomyces in 1% suspension responds to ultraviolet irradiation in the same fashion as Candida.

Of special interest is the sensitivity of the vacuolar membrane to ultraviolet radiation; it is at least as susceptible as the cell membrane. No mode of irradiation has been found which would affect one of these membranes but not the other.

No detailed analysis of the organic constituents released from yeast cells was attempted. The ultraviolet-absorbing material emerges from the cells almost quantitatively under suitable conditions, and an analysis would not add to the existing information on yeast cell constituents. The lack of specificity of the release mechanism raises doubt about attributing⁽²⁾ specific biological significance to the phenomenon.

Summary

Effects of ultraviolet irradiation on yeast cells (Candida utilis) have been studied by ultraviolet photomicrography and by biochemical analysis of ultraviolet-absorbing cellular constituents. The results of these techniques have been correlated.

Much larger doses of ultraviolet irradiation are required to produce morphological changes and altered membrane permeability than to stop the ability to form colonies.

Morphological changes consist of enlargement of the vacuole, followed by a decline in cell size and, eventually, coagulation of cellular protein.

The vacuolar membrane loses its selective permeability in the same fashion as the cell membrane; vacuolar constituents are released into the cytoplasm and thence into the surrounding medium along with cytoplasmic constituents.

The effect of ultraviolet irradiation is not immediate; after a sufficient dose, some time is required for the completion of the release of cellular constituents into the surrounding medium. There appears to be

no selective specificity in the types of compounds released, because at 25°C essentially all material absorbing at 260 m μ is lost by the cells if the exposure to ultraviolet irradiation has been high enough.

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MITOCHONDRIAL SWELLING INDUCED BY THIOPHOSPHATE, 2-MERCAPTOETHYLAMINE, AND AET

John F. Thomson

Several types of mitochondrial swelling have been described by Lehninger *et al.*⁽¹⁾ One of these, produced by thyroxine, orthophosphate, calcium, etc., is readily reversible by addition of ATP and magnesium; another, produced by glutathione and cysteine, is not reversible by such additions.⁽²⁾

Among a series of inorganic phosphates tested for their effect on oxidative phosphorylation, sodium thiophosphate (or phosphorothioate) was unique in that it produced a true uncoupling.⁽³⁾ At a concentration of 0.005 *M*, oxygen consumption was increased by 26%, whereas phosphate esterification was decreased by 28%. It was, thus, of interest to study the effect of this compound on mitochondrial swelling.

The fact that thiols, such as cysteine and glutathione, produced irreversible swelling suggested that this action of these compounds might possibly be related to their radioprotective properties. The observation that poor protective agents such as BAL, thioethanol, and methionine did not induce swelling⁽²⁾ could be construed as support for this view. To test this hypothesis, 2-mercaptoethylamine (MEA) and S-(2-aminoethyl) isothiuronium bromide hydrobromide (AET) were examined for their capacity to induce mitochondrial swelling.

Methods

Mitochondria were prepared from rat liver by gradient centrifugation.⁽⁴⁾ Swelling was studied by diluting suspended mitochondria in 3 ml of a solution of 0.125 *M* KCl and 0.02 *M* tris buffer, pH 7.4, and following the change in optical density at 520 m μ . The temperature was kept constant at 25°C. Sodium thiophosphate (Victor Chemical Works) was used at a concentration of 0.005 *M*. The thiols were tested at several different concentrations. Under the conditions of the experiment, AET rearranged to mercaptoethylguanidine.

Results

Thiophosphate. Figure 48 shows that 0.005 *M* thiophosphate accelerated mitochondrial swelling to approximately the same extent as an equimolar amount of orthophosphate, and that the swelling could be reversed by addition of ATP and magnesium ion. In comparison with other uncoupling agents, thiophosphate thus resembles thyroxine in its effects on mitochondrial swelling, and is unlike dinitrophenol, which inhibits spontaneous swelling.

Figure 48

Mitochondrial swelling induced by 0.005 M thiophosphate and orthophosphate. After 10 minutes (arrow), 15 μ moles of ATP and 10 μ moles of MgCl_2 were added, subsequent readings have been corrected for the increase in volume of the suspending medium.

AET and MEA. Figure 49 shows the effects of 0.01 M concentrations of these two thiols, in comparison with glutathione and cysteine, on mitochondrial swelling. It may be noted that MEA was considerably less potent than glutathione or cysteine, and that AET not only failed to induce swelling, but also delayed spontaneous swelling somewhat. AET had a very slight antagonistic effect on swelling produced by glutathione. Since the response to cysteine concentration has been shown to be biphasic, i.e., 0.001 M was more active than 0.01 M,⁽²⁾ lower concentrations of both MEA and AET were examined. At 0.001 M and 0.003 M, these two compounds were virtually without effect.

Thus, there is apparently no correlation between mitochondrial swelling and protective capacity.

Figure 49

Mitochondrial swelling induced by 0.01 M glutathione, cysteine, 2-mercaptoethylamine (MEA), and AET.

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PHOTOPERIODIC INVESTIGATIONS. PART I. VARIABILITY IN
FLOWERING OF XANTHIUM NEAR CRITICAL DAY LENGTHS

William Chorney

Xanthium is a short-day plant that will initiate flower primordia when subjected to a single dark period longer than that critical for flowering. When given a strong inductive cycle, as for example a dark period exceeding the critical by 3 or more hours, all plants in a given treatment series will reach essentially the same stage of floral development in a given time period.⁽¹⁾ In attempting to develop a bioassay for the "flowering hormone" using Xanthium as the test plant, control plants were maintained for periods of 1 to 2 weeks on a 16-hr day (8-hr night), just above the reported critical day length for maintaining them in the vegetative condition.⁽²⁾ It was observed that some of these untreated plants flowered while others remained vegetative. Experiments were undertaken to determine the variability in our line of Xanthium when maintained for long periods of time close to the critical day length.

Xanthium plants were grown in the greenhouse on an 18-hr photoperiod until they were subjected to the experimental treatment. They were then transferred to a room providing a controlled environment: light of constant quality and intensity of 1200 foot-candles, and a temperature maintained at $75^{\circ}\text{F} \pm 2^{\circ}\text{F}$. There they were maintained on a 16-hr day, light on at 8:30 a.m., off at 12:30 a.m., for periods up to 6 weeks. Periodically some of the plants were dissected to determine the condition of the meristem. They were scored as being vegetative, preinduced or in flower.⁽³⁾

The results of four experiments (Table 32) show the variable flowering response of Xanthium to photoperiods close to the critical. For example, after 28 days, in Experiment 1, 2 plants were vegetative, 6 plants were at preinduced stages and 2 plants were in flower, while in Experiment 2, 4 were vegetative, 2 preinduced and 2 in flower. After another 2 weeks, 5 plants of Experiment 2 were still vegetative, 2 were preinduced and 3 were in flower. In Experiment 4, some plants were in flower after only 14 treatment days; in Experiment 3, in which the plants were on a 16.5-hr photoperiod (7.5-hr night) after 14 days, 5 plants were vegetative, 6 preinduced and 1 was in flower; after 21 days, 1 was vegetative, 6 preinduced and 5 were in flower. In other experiments, plants kept on a 16-hr day in the controlled environment room for 10 days, transferred to the greenhouse on an 18-hr photoperiod and dissected 10 to 12 days later, showed essentially the same variable response. Although most of the plants were vegetative, a few were at preinduced stages, and occasional plants had flower primordia.

TABLE 32

Flowering response of *Xanthium* maintained on a 16-hr photoperiod

Experiment No.	Date	No. of days on 16-hr photoperiod	No. of plants per treatment	No. of plants of			Avg. No. of nodes at beginning of experiment
				Vegetative	Preinduced	In flower	
1	7/2/59	14	10	10	0	0	24
		28	10	2	6	2	
2	10/30/59	14	10	10	0	0	9
		28	8	4	2	2	
		42	10	5	2	3	
3*	2/24/60	14	12	5	6	1	14
		21	12	1	6	5	
		28	12	0	8	4	
4	5/23/60	14	10	3	5	2	12
		24	10	0	9	1	
		36	10	0	5	5	

* These plants were on a 16.5-hr day, 7.5-hr night.

This variability in flowering can probably be attributed to the genetic heterogeneity of the wild population of seed collected in the field and is only observed when plants are given a minimal inductive cycle or are kept on photoperiods near the critical for relatively long periods of time. It appears that at least three strains of *Xanthium* may be present in this seed line. All may have different critical photoperiods for flowering; this would account for the variable response observed.

Recently different lots of *Xanthium* seed have been obtained, and preliminary experiments indicate that they differ in the sensitivities of their responses to photoperiodic induction. One lot requires two consecutive long nights and another a single 11-hr night to initiate flower primordia. For the line of *Xanthium* used in the present experiments a single 9-hr night is sufficient to initiate flowering. The least sensitive of the new lines of *Xanthium* will remain strictly vegetative when grown for 6 weeks on a 16-hr photoperiod, while the other line will reach preinduced stages after 4 weeks.

The extreme variation in flowering response reported here for our *Xanthium* line precludes its use in studies in which plants are subjected to threshold stimuli.

Use of Two-Branched Plants in Studying Flowering in Xanthium

Because of the variation in flowering response of Xanthium, probably due to genetic variability of field-collected seed as described in the first section of this report, it was thought that the effects of threshold stimuli on flowering could be determined more precisely in two-branched plants, by comparing the flowering response of the treated branch with that of its partner control branch. Two-branched plants are obtained by severing the main stem of seedlings above the primary node; this permits the lateral buds in the axis of the primary leaves to become active and produces plants with two branches of identical genetic composition. With selection, plants can be obtained whose branches have developed at the same rate and, therefore, are probably in the same physiological condition. Before two-branched plants could be used experimentally it was necessary to ascertain (1) that both branches of a two-branched plant will develop flower primordia at the same rate when subjected to weak inductive stimuli, and (2) that the flowering stimulus from the induced branch will not affect its partner non-induced branch under the experimental conditions employed.

In the number of experiments in which both branches of a two-branched plant were subjected to weak inductive stimuli, microscopic examination revealed that both branches developed flower primordia at the same rate.

To determine the effect of an induced branch on its non-induced partner branch, one branch of a two-branched plant was given 1 to 4 inductive cycles consisting of a 16-hr night by enclosing it in a light-tight container from 4:30 p.m. to 8:30 a.m. The non-induced branch was maintained on an 18-hr day during the inductive treatment. After induction the plants were grown on the 18-hr photoperiod until they were dissected at 9 to 11 days following the first inductive cycle. Where macroscopic flowers were not present, the floral stages were scored according to Salisbury's scheme: ⁽¹⁾ 0, vegetative; 1 to 3, preinduced stages; 4 to 8, flower primordia present, with the higher number representing the more advanced flowering stages. Theoretically, the stronger the stimulus, i.e., the longer the inductive night, the more advanced the flowering will be. Six plants were used per treatment.

The results (Table 33) show that only after four consecutive inductive cycles will the stimulus from the induced branch affect its partner branch. Since the two-branched plant technique is designed to test weak threshold stimuli, transmittal of the stimulus from one branch to the other is unlikely. In plants that received 1 to 3 inductive cycles only one of 6 receptor branches reached stage 1 of floral development. Since this also occurred in the control plants, i.e., those kept on 18-hour photoperiod continuously, it is not significant. In the plants that received 4 inductive cycles the receptor branches on the average reached stage 2 of floral development.

TABLE 33

Flowering condition of receptor branch when induced branch is given a number of inductive cycles

No. of inductive cycles	Avg. flowering condition of induced branch*	Avg flowering condition of receptor branch*
0	0.2	0.2
1	7.5	0.2
2	m	0.2
3	m	0.2
4	M	2.0

m = a small macroscopic flower that can be seen after one or 2 bract leaves are removed.

M = large macroscopic flower visible without removing any leaves.

*See text for interpretations of these values.

None of the non-induced branches in the experimental time period developed flower primordia. From these results it can be concluded that when working near critical conditions for flowering, or when a weak stimulus is being used to induce flowering, one branch of a two-branched plant can serve as a control for the treated branch. This type of control becomes especially important when studying quantitative induction of flowering, particularly when control plants show some degree of flowering as when plants are kept near a critical day length for relatively long periods of time.

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CYTOLOGICAL OBSERVATIONS OF THE MALE REPRODUCTIVE
ORGANS OF THE MOUSE AND DOG FOLLOWING
ADMINISTRATION OF DEUTERIUM OXIDE

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In recent years there has been a renewal of interest in the effect of isotopic substitution of the natural constituents in organisms. Owing to the availability of relatively large amounts of deuterium oxide and the ease with which various internal concentrations can be maintained, the physiological effects of this isotope have been studied in several plant and animal organisms. These studies have revealed a depressant action on many living systems, presumably due to the greater stability of deuterium-carbon bonding.⁽¹⁾ Previous work with rapidly growing systems, such as tumors and embryos, has described some of these effects of deuterium.⁽²⁻⁵⁾

In previous studies at Argonne National Laboratory, female mice had been placed on 30 atom percent deuterium oxide drinking water for one week prior to mating, and this regimen was continued throughout pregnancy. When the uteri of these animals were examined during the last week of gestation, they contained no viable fetuses although some normal fetuses were found in animals maintained at 25 atom percent deuterium and lower concentrations.⁽³⁾ When males were maintained on 30 percent deuterium oxide and mated with non-deuterated females at two-week intervals, their ability to impregnate the female mice dropped to zero after two weeks.⁽⁶⁾ In addition to the males that were used for mating, others were maintained at the same deuterium level in the drinking water and sacrificed at various time intervals. The reproductive organs of these mice proved to be of such interest cytologically that a more detailed study was undertaken.

Methods

Two series of 100-day-old CF No. 1 male mice were maintained on 30 atom percent deuterium oxide in their drinking water. The deuterium oxide used in this study was triply distilled from stock with a tritium content of less than $0.01 \mu\text{c/g}$.⁽¹⁾ In both series, animals were allowed to wash out the deuterium for limited periods by returning them to ordinary drinking water. In the first series three experimental and two non-deuterated control animals were sacrificed after 5, 9, 15, 20, 26, 30 and 35 days on deuterium.

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Each time a group was sacrificed, two experimental animals from this group were returned to regular water and sacrificed at the next point. The first two animals of this washout group were on 30% deuterium oxide for 5 days and on regular water for 4 days; the next two animals were on 30% deuterium oxide for nine days and off for 6, and so on. Rectal temperatures were taken every five days with a thermistor type temperature indicator.

In the second series, three experimental and two control animals were sacrificed after 30, 35, 40, 45, 50 and 55 days on 30% deuterium oxide. At each interval four additional experimental animals were returned to regular water. Two of these washout animals were sacrificed after two weeks and two after four weeks.

All animals were sacrificed by cervical fracture and the testes were immediately excised and weighed. Liver samples were taken and analyzed for deuterium content by infrared spectrophotometry.*

The testes and epididymises were fixed in Zenker-formol solution, dehydrated and cleared in dioxane, and embedded. Serial sections of each testis were cut at seven microns and mounted six sections per slide. One testis was sectioned up to and through its longest longitudinal axis for quantitative cytological studies which are now in progress. The other was sectioned transversely for qualitative studies of the spermatogenic and spermiogenic stages of the seminiferous epithelial cycle. For these procedures alternate slides were stained with Feulgen - fast-green and the PAS method of Hotchkiss.⁽⁷⁾ The staging of the cycle of the seminiferous epithelium and the stages of spermiogenesis is based on Oakberg's account for the mouse.⁽⁸⁾

Dog gonadal material was available for comparative purposes from a purebred, one-year-old beagle that was maintained on 50% deuterium oxide in the drinking water for six and one-half days, and on 75% D₂O for five and one-half days plus additional deuterium oxide in the food. The dog died on the 13th day when the level of deuterium in the body fluids ranged from 33 to 35%. The testis and epididymis were fixed in Zenker-formol solution, dehydrated in an alcohol series, cleared in methyl benzoate and benzene, infiltrated, and embedded. The sections were cut at seven microns and stained in the same way as the mouse tissues.

Results

In the first series, male mice maintained on 30% deuterium oxide for 30 to 35 days showed a moderate number of "giant multinucleate cells" in the seminiferous tubules. The PAS-stained preparations showed that these giant cells contained nuclei of spermatids in various stages of

* The deuterium oxide analyses were done by Sue E. Mandeville of the Chemistry Division.

development. However, the nuclei in any single giant cell were all in the same developmental stage, which varied from 1 to 6. In no instance was a giant cell observed that contained spermatogonia or spermatocytes. In addition, there was also another type of giant cell which contained elements of late spermatids: complete head-pieces or round globular bodies which are probably nuclear remnants of very late spermatids. Some of the tubules showed a sloughing of cells into the lumina. These exfoliated cells are usually pyknotic and, since no PAS-positive acrosomic material was seen, they undoubtedly represent spermatocytes that were unable to complete division. In none of the preparations did the spermatogonial divisions appear disturbed, for the tubular alignment and numbers of types A, B and intermediate spermatogonia appeared the same as they did in control material. There was no evidence of azoospermia (i.e., lumina containing no mature spermatozoa) in the epididymis, although some of the exfoliative material was present. The giant cells were not seen in any numbers before the 30th day of deuteration, and the non-deuterated control animals showed none of the above abnormalities.

In the second series of mice, a very small number of giant cells appeared in the tubules of the testis after 30 and 35 days' deuteration. The preparations from mice after 40 days showed a large number of giant cells (Figures 50 and 51). The stages of the seminiferous epithelial cycle were completely disrupted and staging of individual tubules was impossible. Very few, if any, immature spermatozoa (very late spermatids) were present in the tubules. The epididymis showed complete azoospermia with the lumina either empty of testicular material (in the caput) or filled with exfoliated cellular debris from the testis (in the cauda) (Figures 52 and 53). Few of the large giant cells were observed to be intact in the epididymis, but some multinucleate pyknotic cells were present. Data from the wash-out animals of both series are incomplete at this time.

The preparations of the dog testis showed severe damage to most of the tubules. Numerous giant multinucleate cells were seen that were similar to those found in the mouse (Figures 54 and 55). The epididymis showed lumina containing immature spermatozoa. This result was expected since the duration of deuteration of the dog precluded azoospermia.

In the first series, no significant differences in the body temperatures of deuterated, washout and non-deuterated animals were apparent from day zero to day 35. During this interval the range in the control group was 97.1°F to 98.5°F and was 97.7°F to 99.5°F in the deuterated mice.

In the first series the distilled deuterium content of the liver samples ranged from an average of 17.4% after 5 days treatment to an average of 19.1% after 10 to 35 days' treatment. (Mice approach the equilibrium concentration in 10 days.)⁽⁹⁾ Data for the second series are incomplete at this time. In the dog the deuterium content in the serum was 20% at day 4, 25.6% at day 7, 35.2% at day 10 and 33.6% postmortem.

Figure 50. Tubule from testis containing several giant multinucleate cells.

Figure 51. Giant multinucleate cell containing early spermatid nuclei.

Preparations of mouse testis and epididymis following administration of 30 atom percent deuterium oxide in the drinking water for 40 days.

Figure 52. Caput epididymis showing empty tubules and one tubule containing exfoliative debris in the lumen.

Figure 53. Cauda epididymis showing exfoliative debris in the lumen of a tubule.

Preparations of mouse testis and epididymis following administration of 30 atom percent deuterium oxide in the drinking water for 40 days.

Figure 54. Tubule containing numerous giant multinucleate cells.

Figure 55. Giant multinucleate cell containing early spermatid nuclei.

Preparations of dog testis following administration of deuterium oxide in the drinking water and food.

The weights of the deuterated testes of the second series appear to be lower than those of the non-deuterated mice, but the data are not extensive enough to show a statistically significant difference.

Discussion and Conclusions

In the two weeks before the onset of infertility, there is a period of initial fertility, which can be attributed to the continued development of spermatids and spermatozoa. Infertility appears to be a result of abnormal spermiogenesis and not of aspermia, which would be a result of a spermatogenic block, a situation not observed in our preparations. Although there is considerable exfoliation of spermatocytes in the deuterated animals, enough spermatogenic cells remain to overcome this loss. If the sterile period resulted from damaged spermatogonia, as in irradiated testes, our preparations would not show dividing spermatogonia and spermatocytes. What is more important, irreversibly damaged spermatogonia would elicit the classic irradiation picture of "empty" seminiferous tubules containing only Sertoli cells. This was never observed in our preparations of testes from deuterated animals.

As to why the early and very late spermatid remnants form a symplasm, we can only speculate that the integrity of the cell membrane is destroyed after a period of time on deuterium, and this change results in the coalescence of the formerly individual spermatid nuclei into a common pool of cytoplasm. Whether the giant "cells" then form a new cell boundary in the form of a membrane is not known and will require electron microscope studies. These might enable us to establish them as true cells bounded by a cell membrane or to classify them as syncytia or as agglomerations of cellular debris.

The fact that the giant multinucleate cells contain spermatid nuclei and that none were observed to contain spermatid precursors makes it unlikely that these giant cells form via several mitotic divisions, since haploid spermatids of higher organisms are not known to undergo mitosis.

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STUDIES ON THE EFFECTS OF DEUTERIUM OXIDE

VIII. The Effect of Deuterium on the Reproductive Potential of Male Mice

Asher J. Finkel and Dorice M. Czajka

The manner in which replacement of hydrogen with deuterium in the body fluids affects the abilities of female mice to reproduce has recently been summarized.⁽¹⁾ While the administration of 25% D₂O in the drinking water led to an apparent decrease in the number of pregnancies carried close to term and to an increase in the incidence of wholly non-viable litters, further analysis revealed that these findings reflected certain intra-uterine and postpartum events. Examination of uterine contents late in pregnancy showed that neither the incidence of pregnancy in young adult females nor the mean number of implanted embryos per pregnant female was correlated with the length of time the dams had been deuterated. On the other hand, an increase was seen in the nonviability of fetuses examined in utero late in gestation, and this reduction of fetal viability was greatest when the dams had been substantially deuterated at the time of implantation of the fetuses at the fifth day after mating.

The current report summarizes a corresponding series of experiments in which deuterated male mice were mated at regular intervals with nondeuterated females. Viability and modes of nonviability of the fetuses were studied by examination of the intra-uterine contents of the females. These results were then related to the duration of deuteration of the males.

Methods

Four groups, each containing 15 100-day-old CF No. 1 male mice were placed on drinking water that contained 0, 10, 20 or 30% D₂O in the drinking water. After a single control mating period prior to the onset of deuteration, the males were housed overnight with two or three females in estrus. These female mice were 100 to 150 days old and were selected from a large, continuously changing population by vaginal smear.⁽²⁾ At the time of the second mating period, the males were in equilibrium with the D₂O in the drinking water, and the separate groups presumably had the following tissue fluid concentrations of deuterium: 0, 6.5, 13.4, and 20.0%, respectively.⁽³⁾

The female mice were sacrificed 17 to 18 days after mating, during which time they were maintained on ordinary water. The numbers of grossly normal fetuses were tabulated along with the numbers of autolyzed embryos (late fetal deaths) and of deciduomata (early fetal deaths). In addition, the corpora lutea of each ovary were counted to determine, by difference, the number of preimplantation deaths.⁽⁴⁾

The male mice drinking 30% D₂O were returned to regular water after 42 days because of two completely sterile mating periods. They were remated every two weeks thereafter until reappearance of pregnancy in the females. The animals drinking 10 and 20% D₂O were returned to regular water after having been on D₂O intake for 70 days, and they were also rebred fortnightly during this washout period.

A second series of male mice was maintained on the same set of D₂O drinking water arrangements in a parallel study, and animals were sacrificed at intervals in order to provide material for a cytological study of spermatogenesis. A preliminary summary of this part of the study is reported elsewhere.⁽⁵⁾

Results and Discussion

The tabulation of the various intra-uterine findings is given in Table 34. The total number of fetuses is the sum of the normal fetuses, the early fetal deaths (deciduomata), and the late fetal deaths (autolyzed fetuses). The number of preimplantation deaths is determined by the difference between the number of corpora lutea and the total number of fetuses (implantation sites).

The nondeuterated controls had a pregnancy rate ranging from 13.3 to 46.7% under the conditions of these experiments. Preimplantation deaths accounted for from 4.2 to 11.9% of the number of corpora lutea. Normal fetuses comprised 77.8 to 88.8% of the number of corpora lutea, with early fetal deaths amounting to 5.5 to 11.1%, and late deaths 0.0 to 3.3%. The ranges of the percent of normal fetuses and of the percent of preimplantation deaths are shown in the shaded bands in Figures 56 to 58.

When the males were kept on 10% D₂O, their reproductive potential was apparently unimpaired (Figure 56). Except for sporadic departures at 0 and at 70 days, the percentages of normal fetuses and of preimplantation deaths, in relation to the number of corpora lutea, was close to or within the ranges obtained with nondeuterated males. The incidence of pregnancies was also similar to that of the control mice.

Deuterium replacement of hydrogen at a level of 20% in the drinking water led to definite impairment of reproductive ability of the males between the 14th and the 28th days of deuteration (Table 34, Figure 57). By the 28th day there was a definite drop in the relative incidence of normal fetuses and an increase in the percentage of preimplantation deaths. The incidence of pregnancy, however, was not affected, until the 56th day. With the return of the male mice to ordinary water on the 70th day, there was a gradual recovery in the reproductive ability of the males so that the normal range was reached after 35 days.

TABLE 34

Intra-uterine contents of mice mated with deuterated males.

D ₂ O Group	Mating day	No. of Mice		No. pregnant	No. of corpora lutea	No. of preimplan- tation deaths	Total No. of fetuses	No. of normal fetuses	No. of early fetal deaths	No. of late fetal deaths
		Male	Female							
30% D ₂ O	0	15	30	11	123	24	99	90	8	1
	14	15	31	3	37	1	36	32	4	0
	28	15	45	0	0	0	0	0	0	0
	42	14	42	0	0	0	0	0	0	0
Return to 100% H ₂ O	7	14	42	0	0	0	0	0	0	0
	21	14	42	0	0	0	0	0	0	0
	35	14	42	2	26	1	25	25	0	0
	49	14	42	6	73	5	68	61	7	0
20% D ₂ O	0	15	30	10	114	5	109	101	8	0
	14	15	30	12	129	6	123	93	13	17
	28	15	45	17	203	113	120	103	5	2
	42	14	42	11	115	42	73	64	7	2
	56	14	42	3	38	14	24	21	3	0
	70	14	42	7	83	13	60	52	5	3
Return to 100% H ₂ O	7	14	42	3	41	13	28	23	3	2
	21	14	42	12	139	32	107	101	6	0
	35	14	28	6	77	14	63	59	4	0
	49	14	28	5	99	5	94	90	4	0
10% D ₂ O	0	15	30	3	30	6	24	20	4	0
	14	15	30	9	106	8	98	79	15	4
	28	15	45	12	144	11	133	107	25	1
	42	14	42	12	147	6	141	127	12	2
	56	14	42	4	44	10	34	25	6	3
	70	14	42	5	65	3	62	51	10	1
Return to 100% H ₂ O	7	14	42	4	50	5	45	40	2	3
	21	14	42	1	11	1	10	9	1	0
	35	14	28	4	53	3	50	45	5	0
	49	13	26	6	74	9	65	54	11	0
0% D ₂ O	0	15	30	14	158	18	140	128	11	1
	14	15	30	14	181	16	155	146	5	4
	28	15	45	14	181	12	169	154	12	3
	42	15	45	9	109	13	96	86	9	1
	56	15	45	14	170	9	161	145	11	5
	70	15	45	17	192	8	184	160	20	4
	84	15	45	13	171	19	152	133	19	0
	98	15	45	6	81	6	75	65	8	2
	112	14	28	7	91	10	81	73	5	3
	126	14	28	8	107	4	103	95	7	1
All groups 0% D ₂ O	0	60	120	38	425	53	372	339	31	2

Figure 56

Incidence of normal fetuses and of preimplantation deaths (both as percentages of number of corpora lutea) in female mice mated with male mice that had been deuterated with 10% D_2O in the drinking water. Shaded areas represent the corresponding ranges for nondeuterated control mice.

Figure 57

Incidence of normal fetuses and of preimplantation deaths (both as percentages of number of corpora lutea) in female mice mated with male mice that had been deuterated with 20% D_2O in the drinking water. Shaded areas represent the corresponding ranges for nondeuterated control mice.

Figure 58

Incidence of normal fetuses and of preimplantation deaths (both as percentages of number of corpora lutea) in female mice mated with male mice that had been deuterated with 30% D_2O in the drinking water. Shaded areas represent the corresponding ranges for nondeuterated control mice.

When the male mice were on an intake of 30% D₂O there was a striking reduction of reproductive ability after 14 days. Since the number of pregnancies dropped to zero by the 28th day mating period, the other indices have little meaning. The absence of any corpora lutea indicates that the males may have been affected in their mating behavior and may not have copulated in all cases. If they had mated, and if the sperm were defective as a result of deuteration, pseudopregnancy would have developed, and corpora lutea would have been seen up through the 12th day. Since our examination occurred on the 17th or 18th day, the absence of corpora lutea does not distinguish between pseudopregnancy and failure to mate. On the other hand, the results with 20% D₂O point to an effect on spermatogenesis that becomes manifest some time between two and four weeks after the institution of deuteration of the male mice. This effect may well occur in the more highly deuterated male mice, but they may also be adversely affected in their mating behavior so that the effect on spermatogenesis may be masked. In any case, the effect on reproductive ability is reversible, for after the 30% D₂O in the drinking water was replaced with ordinary water, matings occurred, pregnancies resulted, and the incidence of normal fetuses returned to normal. These events, signifying recovery, are evident from the results of matings at the 35th day and occur again with matings two weeks later.

The results obtained in these experiments are generally consistent with those obtained in a different way by Hughes, Bennett and Calvin,⁽⁶⁾ who studied male mice that had been deuterated for two or three week periods. The results of both groups of investigators are in agreement in pointing to a deuterium effect on spermatogenesis that probably occurs at the time of development of the secondary spermatocytes or spermatids. More precise delineation of the effect of D₂O on spermatogenesis will have to depend on the results of work in progress dealing with the cytological appearance of spermatozoa and their precursors.⁽⁵⁾

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CELL RENEWAL IN LEUKEMIC STATES IN MAN: A PRELIMINARY REPORT

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These studies were initiated in an attempt to establish certain basic parameters of the renewal of cells in acute and chronic myelogenous leukemia. The developmental sequence in marrow and the transit in blood are being evaluated autoradiographically in leukemic patients receiving tritiated thymidine.

The initial study was performed on three patients, one with chronic myelogenous leukemia, another with acute myelogenous leukemia and a third hematologically normal. Tritiated thymidine in a dose of $100 \mu\text{c/Kg}$ was injected intravenously. Bone marrow samples were aspirated from the sternum under local anesthesia and collected into an equal volume of heparinized human plasma. Blood samples were taken from the antecubital vein. Autoradiographs were prepared with stripping film and stained with a modified Giemsa Stain.⁽¹⁾ Since cells capable of proliferation are circulating in the peripheral blood in leukemia, it is essential to characterize the early behavior of these cells in blood as well as in marrow. The chronic leukemic patient had been diagnosed 10 months earlier and had received no X-ray or chemotherapy prior to this study. The leucocyte count was $150,000/\text{mm}^3$ with a peripheral differential cell count of polymorphonuclear cells, 20%; stab, 18%; metamyelocyte, 13%; myelocyte, 41%; promyelocyte, 3%; myeloblast, 2%; lymphocyte, 3%. The patient with acute leukemia had a peripheral leukocyte count of $76,000/\text{mm}^3$ with approximately 90% myeloblasts. The hematologically normal patient was a 70-yr old male with a leucocyte level of $8500/\text{mm}^3$ and a normal differential cell count.

The course of events in marrow and blood is shown in Figure 59. The percent initial labeling of the proliferative cells in the bone marrow and blood of the chronic leukemic patient suggests that DNA synthesis time represents the same proportion of the cell cycle as in the normal. The data suggest further that the DNA synthesis time must be the same as in the normal in view of the similar build-up of labeled cells in both the proliferative pool and in the metamyelocytes. If the DNA synthesis time were shorter in the chronic leukemic patient, the turnover time would also be shorter with a resultant faster rate of increase of succeeding classes of labeled cells, assuming that the time spent in such classes was not altered. Labeled metamyelocytes appear and increase at the same rate, and labeled segmented neutrophils are evident on the fifth day in both the normal and leukemic patients.

Figure 59

Temporal distribution of labeled myeloid cells in marrow. The values are based on a differential analysis of a minimum of 2000 cells on each sample.

Thus, there is no indication of an altered marrow transit time for the differentiating cells. On the fifth day there is a higher percentage of labeled segmented neutrophils in the chronic leukemic than in the normal patient. Since proliferative cell types were labeled initially in the peripheral blood, this may possibly be attributed to the absence of a marrow sojourn for part of the developing segmented cells. In the dog, the mean time for segmented neutrophils in marrow was shown to be about 15 hrs,⁽²⁾ and this may even be somewhat longer in man. Although the data are still incomplete, there is no evidence of a defect in neutrophil removal from blood based on the final disappearance of the more heavily labeled cells in the normal and leukemic patients as shown in Figure 60.

Figure 60

Labeled segmented neutrophils in peripheral blood. The values are based on enumeration of a minimum of 2000 neutrophils on each sample.

It will be recalled that the blood neutrophil concentration of the latter was some six times that of the former. These studies have, thus far, failed to disclose any sharp differences in chronology of neutrophil development in marrow and in neutrophil disappearance from blood in chronic leukemia.

Turning to acute leukemia, it will be noted in Figure 59 that only a few percent of cells are labeled initially in marrow and blood smears. It is difficult to assess the significance of this since one is comparing all proliferative cells in the normal with a population of proliferative cells in the acute leukemic consisting primarily of myeloblasts. Hence, a recent study has been undertaken to define as precisely as possible the cell cycle of the myeloblast in normal and leukemic states. This involves single and repeated injections of tritiated thymidine and analysis of marrow smears prepared from spicules as well as from aspirated fluid. The first investigations suggest a similar initial labeling of myeloblasts in a hematologically normal patient and in a patient with acute leukemia. Eighteen percent of myeloblasts were labeled in marrow obtained from spicules in the normal and 22% in the acute leukemic patient. Only 9% of leukemic myeloblasts were labeled in the marrow aspirate, and 3% in blood. Further comparisons will be made to establish the possible significance of such differences between aspirate and spicule.

In two patients with acute leukemia the mean grain count of 100 myeloblasts each was determined at intervals after a single injection of tritiated thymidine. The mean grain count did not decrease during the first four hours after injection. Therefore, the post-synthetic (G_2) and mitotic intervals must be at least 4 hr in duration. The mean grain count is decreased by about 40% by 12 hr, which suggests a maximum DNA synthesis time of perhaps 8 hr. From the percent of cells labeled initially and the approximation of the DNA synthetic period, the generation time for the myeloblast (spicule preparation) in the acute leukemic patients is estimated to be 30 to 35 hr. Since this represents a crude approximation, studies are contemplated in which tritiated thymidine will be infused at frequent intervals in order to establish the myeloblast cycle and to determine whether all myeloblasts proliferate in the acute and chronic leukemic states.

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LOSS OF MUTAGENIC POTENTIAL IN BACTERIA

H. E. Kubitschek and H. E. Bendigkeit

In chemostat cultures some latent mutants for resistance to the bacteriophage T5 fail to express the mutant phenotype when cell growth rate is limited with glucose or lactic acid.⁽¹⁾ We also have recently observed this phenomenon in phosphate-limited cultures. The fraction of latent mutants reaching expression appears proportional to growth rate. Failure to express is due, presumably, to cell death or to an interruption or reversal of the mutagenic process.

We have obtained evidence that the loss of latent mutants in *E. coli* B is a temperature-dependent, first order biochemical reaction, and is not due to the dilution of an intracellular mutagen. Our evidence implies that latent mutants are primarily cells in which the genetic apparatus has been modified as a result of the application of the mutagen; these cells might be genotypic mutants.

When a culture of *E. coli* B is limited with glucose, and then allowed to become stationary before exposure to ultraviolet light, the latent mutant concentration first increases after nutrient is again supplied, then decreases again as latent mutants become expressed, die, or revert to wild type. Although the fraction expressed is dependent upon growth rate, loss of latent mutants appears to be growth rate independent. Figure 61 shows the loss of latent mutants in cultures with several different generation times at 37°C. It may be seen that the rate of loss appears to have the kinetics of a first order reaction with a half-value of about $12\frac{1}{2}$ hr. If the rate of loss is not dependent upon the rate of division of cells in the chemostat, it cannot be dependent upon the concentration of a hypothetical intermediate mutagenic substance within the cell. Concentration independence implies that the mutational potential is reproducing along with the cell, most plausibly by association with the genetic apparatus.

Figure 61
The loss of latent mutants in chemostats at 37°C; growth limited with glucose.

Preliminary measurements of the rate of loss of latent mutants in samples removed from chemostats and held at constant temperature also indicate an exponential loss, with rates decreasing as the temperature is reduced (Table 35). The energy of activation calculated from these data is approximately 25 kcal.

TABLE 35

Time, $T_{1/2}$, for loss of half of the
concentration of latent mutants
as a function of temperature

Temperature, °C	$T_{1/2}$, hr
5	1100
18	400
25	60
37	13

Reference

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A SEARCH FOR RADIATION-INDUCED RECESSIVE MUTATIONS IN THE MOUSE

Herman M. Slatis

Most of the studies of radiation-induced mutations in the mouse have concentrated on the production of mutations at specific (predetermined) loci. In contrast with this, the present study is directed toward finding mutations that have occurred at any locus, provided that some aspect of each mutation is detectable by the techniques employed.

Male C57Bl/6 mice were given an X-ray dose of 1000 r to the posterior portion of the body. After three months, these males were placed with female mice and any males that proved to be fertile were given a number of females. The daughters of this cross were destroyed, while the sons were used in further matings with unirradiated females. In these second-generation crosses, the sons were destroyed and the daughters crossed back to their own fathers. The result of this process was to produce a generation of mice which might possibly be homozygous for genes that were mutated by the heavy irradiation of their ancestor. A control group of mice was developed by a similar inbreeding schedule, but without any irradiation in their ancestry.

Thus, this experiment will be comparing two groups of mice which have similar inbreeding in their immediate ancestry and which differ from each other only in that the experimental group is descended from an irradiated individual. The characteristics that are being examined among these mice are viability, sex ratio, and visible changes in the phenotype.

Over 300 litters and more than 2000 mice were observed both in the experimental and the control groups. Infant mortality in this strain has been extremely high, with about 50% of the liveborn animals dying before weaning. This and other viability differences between the two groups of mice have been slight, but favorable to the mice with irradiated ancestry. Analysis of the differences observed is complicated by intralitter factors, which account for a large proportion of the deaths prior to weaning. Similarly, survival to time of death of weaned animals is highly non-random in that the animals that are caged together have a strong tendency to die within a short period of time. A statistical description of these events is being formulated. No "visible" mutations appeared among the group with irradiated ancestry. Among the control animals, one line showed a severe, fatal malformation of the head in several animals. This appears to have been due to a newly occurring, spontaneous mutation.

The basic principles of this experiment are being repeated with a strain which has a much lower infantile death rate, the Argonne strain of C3H mice. This should permit a more satisfactory observation of any changes in neonatal death rate. The effect of caging on the time to death in mature mice is not easily overcome, so this observation will not be made on the new series of mice. Not carrying the mice to the time of natural death will permit a more efficient utilization of space, so that far larger numbers of newborn mice can be observed.

TRACHEA-BROKEN, AN UNUSUAL MUTANT IN
DROSOPHILA MELANOGASTER

Herman M. Slatis, Gerald Schuytema, and James E. Trosko*

The larvae of Drosophila melanogaster derive their oxygen through branches of a pair of large tracheal trunks which can clearly be seen through the dorsal surface of living larvae. In most stocks of this species, a very small proportion of the larvae will show an interruption of these main tracheal trunks. One stock which is being used for a number of other purposes has shown a greatly increased frequency of this abnormality. The abnormality has not been shown to have an effect on the viability of affected larvae. This condition is unusual in that most mutants having morphological effects on larvae are lethal.

The frequency of observation of the abnormality in a highly inbred stock varies greatly with the culture conditions. Only 1.7% (18/1081) of the larvae were affected among the progeny of single pairs raised at 25°C and 2.6% (27/1054) were affected among larvae grown in mass cultures. These same conditions of crowding gave 17.0% (262/1540) and 10.0% (501/5011), respectively, at 16°C. Mass cultures at 16°C were chosen as the standard for further investigations.

The inheritance of the condition was checked by a cross with a stock of vestigial winged (vg) flies, which only rarely are affected (0.7%, 20/2799). The F₁ of this cross showed only 0.2% (10/4803), indicating that a recessive component is responsible for the condition in whole or in part. The F₂ flies showed agreement with a theory that the condition is largely determined by a single recessive gene, with 2.5% expected and 1.8% (123/6719) observed. However, the backcross, which should have given a frequency of 5.0% affected, gave only 1.7% (87/5028). Some further studies suggest that a third chromosomal gene is largely responsible for the condition, but this has not yet been definitely ascertained.

Larvae with two and three breaks in the tracheae have also been observed, and accurate classification of 5788 larvae for the position and number of breaks shows good agreement with the distribution of multiple breaks expected on the theory that multiples occur at random. In addition, it can be shown that double breaks do not occur adjacently more often than would be expected on the basis of chance alone.

*Student Aide, Summers, 1959, 1960.

PROGRESS REPORT: DISEASES OF LABORATORY ANIMALS

Ectromelia Infection of Mice

Robert J. Flynn

Ectromelia, a virus disease of mice, occurs in both a chronic and an acute form. The chronic or cutaneous form resembles all other pox diseases. It is characterized by typical pox-like dermal lesions and a varying mortality, and is highly contagious. The acute form is characterized by a puffed face, conjunctivitis, necrotizing hepatitis, enlargement of the spleen, and an early, high mortality. Since the animals die before the dermal lesions appear, this form is not as contagious as the other.

The first confirmed epizootic in this laboratory occurred during December 1958 and January 1959. Except for one subsequent outbreak in March 1959 involving only one cage, the animal quarters remained apparently free of the disease until December 1959 when another severe epizootic began. Subsequent to this, two other smaller outbreaks have occurred.

During the above-described epizootics, observations regarding the methods of diagnosing this disease were made. In one instance, two groups of animals from cages displaying a recent high mortality were killed. The symptoms and lesions observed were tallied and correlated with the results obtained by inoculating susceptible animals. (See Table 36). Clinically, 8 of 14 animals showed either a puffy face or conjunctivitis, but only one showed skin lesions. Internally, the majority showed enlarged spleens. Liver necrosis was observed in only two animals macroscopically, but in 11 of 14 animals microscopically. Five of the animals disclosed pathognomonic inclusion bodies; nine were positive by animal inoculation test.

In another instance, the results obtained by inoculating susceptible animals were compared with those obtained by inoculating immune mice and subsequently titrating their sera for hemagglutination-inhibiting (HAI) antibodies. Assuming that an HAI titer of 40 or more is positive, in 16 trials 9 were positive serologically and 9 were positive by animal inoculation. In all but two of these trials the results for both tests were identical. In one of these two, the animal inoculation test was positive and the serology negative; in the other, the converse was true.

Observations on the control of ectromelia were also made. The IHD-T vaccinia virus furnished by the Mouse Pox Service Laboratory (Cancer Chemotherapy National Service Center, National Cancer Institute,

TABLE 36

The symptoms and lesions observed in two groups of naturally infected mice correlated with the results of test animal inoculations

	Clinical observations			Gross pathology		Micropathology			Animal inoculations* test
	Puffed face	Conjunctivitis	Pox lesions	Liver necrosis	Spleen enlarged	Liver necrosis	Liver inclusions	Spleen necrosis	
Group 1-1	-	+	-	+	-	+	-	+	-
2	-	-	-	-	-	+	-	-	+
3	-	-	-	-	+	+	-	-	-
4	-	+	-	-	+	-	-	-	-
5	-	+	-	-	+	+	-	-	+
6	-	-	-	-	+	-	-	-	-
7	+	-	-	-	-	-	-	-	+
8	+	+	-	+	+	+	+	-	+
9	+	-	-	-	+	+	-	-	(not done)
Group 2-1	-	-	+	-	+	+	+	-	+
2	-	+	-	-	-	+	+	+	+
3	-	-	-	-	+	+	+	+	+
4	-	+	-	-	+	+	+	-	+
5	-	-	-	-	-	+	-	-	+

*Both unvaccinated (susceptible) and vaccinated (immune) test animals were inoculated with material from diseased animals; + = susceptible animals dying within 7 days after inoculation but immunized animals surviving.

National Institutes of Health) was found to be extremely effective. Both major epizootics were brought under control within two weeks following the vaccination of all susceptible mice.

That sanitation and quarantine without vaccination were not sufficient to stop an epizootic was noted during the second major outbreak. Because in this outbreak a high mortality was first observed in a room in which all the mice had been vaccinated, ectromelia was not immediately suspected, and a revaccination program was not instituted until after a positive diagnosis was made. During the delay, in spite of rigid sanitation and quarantines, the disease spread throughout the initially affected room and to four other rooms.

Following this outbreak, tests were made to determine if the transplantable tumors used in this laboratory were infected with the ectromelia virus. Susceptible mice were inoculated with filtrates of five different tumors. Four of the five groups of test animals died of ectromelia. The uninoculated control animals died also. When the experiment was repeated none of the animals died. These results indicated that none of the tumors was infected. They also dramatically showed that ectromelia remained endemic in our animal quarters.

Apparently, once animal quarters become infected with ectromelia, it is imperative that all susceptible animals be vaccinated and revaccinated. Unfortunately, the duration of immunity conferred by vaccination is not known. It was originally assumed to be life-long; however, the second epizootic showed it to be somewhat less than a year and perhaps only 4 to 6 months. Therefore, a study was initiated to ascertain the length of time vaccinated mice are refractory to infection with ectromelia virus. However, owing to inadequate facilities for effective isolation, it was not possible to prevent exposure to the natural disease and the study was terminated prematurely.

From these studies and observations it was concluded that ectromelia cannot be positively diagnosed clinically or by observing gross lesions because the only consistent conditions, conjunctivitis and megosplenaly, are non-specific. A more accurate diagnosis can be made microscopically particularly if the pathognomonic inclusion bodies are present. A positive diagnosis can be made by inoculating susceptible animals with suspicious material and comparing the resulting mortality to that of inoculated immune animals; or by inoculating immune animals and observing the subsequent hemagglutinin-inhibition antibody titer. The correlation between the latter two methods is excellent.

It was also concluded that an ectromelia epizootic can be effectively controlled by vaccinating all susceptible animals. Sanitation and quarantine alone are ineffective. Further, once animal quarters become infected, it is imperative that all susceptible animals be vaccinated and revaccinated periodically. Unfortunately, the length of time vaccinated mice are refractory to infection with ectromelia virus remains to be determined.

PROGRESS REPORT: DISEASES OF LABORATORY ANIMALS

Pseudomonas Infection of Mice

Robert J. Flynn

Pseudomonas infection is rapidly fatal for irradiated mice. When encountered, it significantly modifies the course of radiation sickness by greatly decreasing the survival time.^(1,2) Therefore, during the latter part of 1959 when the mean survival time of acutely irradiated (700 r X-rays, an LD₁₀₀) CF No. 1 mice raised in this laboratory dropped from 13.81 ± 0.39 days to 6.01 ± 0.29 days, *Pseudomonas* infection was immediately suspected. This was confirmed by bacteriological studies in which *Pseudomonas* organisms were isolated from more than 90% of the animals.

It was concurrently observed that the survival time of these mice following inoculation with EO(H) ascites tumor was shorter than that of mice of the same strain obtained from Carworth Farms. The relationship of this observation to *Pseudomonas* infection is not known, but the possibility exists that the tumor inoculation provides sufficient stress to activate latent *Pseudomonas* infections in the recipient mice. This possibility is being tested experimentally.

Although the mice raised in this laboratory had been repeatedly checked for *Pseudomonas* infection with consistently negative results, it became clear that the incidence of this organism in our mice was quite high, and that the method being used for detection was inadequate. The essential prerequisite for eliminating this organism from the breeding stocks involved a reliable means of detection; consequently, efforts were directed toward this problem. The present report summarizes variations in methodology which have greatly increased the accuracy of the detection system. Also, reported are the results of a preliminary experiment in which X-irradiation is used in an attempt to confirm the bacteriological results.

Review of Culture Methods

The method that had been employed to test all breeding stock was that of Wensinck *et al.*⁽²⁾ In this method, tubes of glycerol broth* were inoculated with water from the drinking bottles, incubated 24 hr, and then subcultured on glycerol agar** for 24 hr. Positive cultures exhibited

*Glycerol broth consists of 1% (w/v) glycerol, 1.5% Difco proteose peptone, 0.04% K₂HPO₄, 2% MgSO₄·7H₂O, and 0.001% FeSO₄. The pH is adjusted to 7.5 before sterilization at 120°C for 15 minutes; the medium is filtered and sterilized again at 110°C for 10 minutes.

**Glycerol agar is prepared by the addition of 1.5% (w/v) Difco agar to glycerol broth.

the typical blue-green pigment and characteristic odor. Unfortunately, in our laboratory the subculturing from broth to agar was discontinued on the assumption that it was unnecessary. The seriousness of this error can be deduced from the results shown in Table 37. The accuracy of 24 hr culturing in glycerol broth alone is 1.1%. When the broth is incubated for 72 hr and subcultured on glycerol agar, the accuracy is 98.9%.

TABLE 37

Comparative methods of culturing water bottles from known infected cages

Medium	Tetrathionate broth				Nutrient broth			Glycerol broth				Composite of all methods
Hours of incubation	24	48	72	96	24	48	72	24	48	72	72 GAP**	
No. positive/ No. tested	14/89	44/89	56/89	70/82	10/41	22/41	29/41	1/89	19/89	70/89	88/89	89/89
Percent positive	15.7	49.4	62.7	85.4	24.4	53.6	70.8	1.1	21.3	78.4	98.9	100.0

*As indicated by production of green pigment.

**GAP, glycerol agar plate.

Other methods of testing were also attempted, and the results are shown in Table 37. Nutrient broth (Difco) was checked since it is frequently used by others for this purpose. Although it was the most accurate of the methods used at the end of 24 hr, its final accuracy of 70.8% at the end of 72 hr fell short of the other methods. Incubation beyond 72 hr did not increase the incidence of detection.

Tetrathionate broth (Difco), a selective enrichment medium used in this laboratory in the isolation of *Salmonella* was incidentally found to have value in the detection of *Pseudomonas*. If allowed to stand for 96 hr, it was superior to both glycerol broth and nutrient broth; however, the maximum accuracy of 85.4% could not be increased by incubating beyond 96 hr.

Check of Culture Methods by X-irradiation (Preliminary)

Two mice from each of 11 cages found to be infected with *Pseudomonas* by glycerol broth/glycerol agar tests of the drinking water, and two mice from each of 11 cages found to be free of infection, were given 700 r of X-rays. Of the infected mice, 18 of 22 (82%) died before the tenth day after irradiation and the mean survival time was 6.58 ± 0.34 days, whereas none of the non-infected mice died before the tenth day.

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BACTERIAL ENDOTOXINS AND THE RADIATION SYNDROME

E. John Ainsworth

Lipopolysaccharide complexes (endotoxins) derived from gram-negative microorganisms, or intact organisms in the form of a vaccine, are known to decrease acute mortality in X-irradiated mice.⁽¹⁻³⁾ Endotoxin protection is most effective when the material is given 24 hr before irradiation, but significant postirradiation protection may be demonstrated at radiation doses below the LD₁₀₀ level. The mechanism of endotoxin protection is thought to involve stimulation of the bone marrow and induction of hematopoietic recovery, induction of a state of hyperactivity and perhaps proliferation of the sessile elements of the reticuloendothelial system, and an increased titer of bactericidal and opsonic factors in the serum. The present report summarizes experimental results concerning various factors that modify the therapeutic effects of endotoxins in X-irradiated animals, and also information on the comparative protective effects of endotoxins in X-irradiated, γ -irradiated, and neutron-irradiated mice.

The responses elicited by endotoxins are nonspecific in the sense that qualitatively comparable responses are produced by essentially all gram-negative endotoxins. Consistent with this is the observation that endotoxins from four genera of bacteria, prepared by somewhat different extraction procedures, decrease radiation mortality in mice. In contrast to these results, however, are two separate reports involving a commercial *Pseudomonas* lipopolysaccharide which appears to have all of the properties of classical endotoxins but produces no mortality reduction in X-irradiated animals.^(4,5) This material has been re-examined in our laboratory and has been found to be effective under certain conditions. The treatment regimens used by others involved multiple injections given before irradiation, or a single intraperitoneal injection of approximately 0.4 μ g given 24 hr before irradiation. These negative findings were confirmed in CF No. 1/Anl mice with the above treatment regimens. However, by increasing the endotoxin dosage and giving a single injection by the intravenous route 24 hr before irradiation, significant reduction of mortality was produced by either 0.8, 2.0 or 50.0 μ g of *Pseudomonas* endotoxin. The highest dosage used, 50 μ g, resulted in a 93% reduction of 30-day mortality at 700 r. At the 2.0 μ g endotoxin dosage a 77% mortality reduction was produced by intravenous injection, whereas intraperitoneal or subcutaneous injection was ineffective. During the course of some of these studies over a period of several months there was a 50% decrease in mean survival time of irradiated CF No. 1/Anl mice. This was related to the emergence of *Pseudomonas* organisms, which were the principal cause of terminal bacteremia. The altered bacteriological situation resulting from a change in the gut flora of the experimental animals was accompanied by

a complete loss of the therapeutic value of endotoxin; treatment that previously reduced mortality by 93% was without effect. In consequence the use of CF No. 1/Anl mice was discontinued and CF No. 1 mice from the commercial producer were used in all subsequent experiments.

Protection experiments have also been conducted using substances other than gram-negative endotoxins. Although endotoxins differ in many respects from the so-called enterotoxins of certain gram-positive bacteria, some pharmacological properties, such as pyrogenicity, leucocyte mobilization, and promotion of an increased resistance to heterologous infection, are shared. Results presented in Table 38 indicate that gram-positive materials in the form of a polyvalent Streptococcus-Staphylococcus vaccine (SSV) shares with Typhoid-Paratyphoid vaccine (TAB) the property of decreasing radiation mortality. SSV is less effective in this regard than TAB, and the protective component appears to be thermolabile, since boiling for one hour decreases the protective potency.

TABLE 38

Effect of bacterial vaccines on 30-day mortality in
CF No. 1 mice irradiated with 700 r X-rays

Treatment	No. mice	Mortality, %
SSV, 0.1 ml*	46	50
SSV, 0.1 ml, 100°C	33	72
SSV, 0.2 ml	16	56
TAB 0.05 ml**	32	15
Irradiated controls - no treatment	32	96

*SSV, Streptococcus-Staphylococcus vaccine; each ml contains 8×10^9 organisms

**TAB, Typhoid-Paratyphoid vaccine; each ml contains 1.5×10^9 organisms

Studies on the protective effects of endotoxin have been extended to mice irradiated with Co^{60} γ -rays or fission neutrons at the CP-5 reactor. Survival time of neutron-irradiated mice was increased by endotoxin treatment, but mortality was not altered. This may be attributable, in part at least, to the fact that many of the animals die during the first post-irradiation week before effects on hematopoietic recovery can be manifest. The results, summarized in Table 39, show that at the LD_{95-100} level, treatments which are effective in X-irradiated mice are either less effective or ineffective in γ - or neutron-irradiated mice. The surprising difference in the effectiveness of endotoxin in X- and γ -irradiated mice was confirmed in other experiments in which the γ -irradiation was performed in the

high-level gamma room in Building 202. These results, shown in Table 40, indicate that no protection was afforded by *Pseudomonas* endotoxin and this treatment even increased mortality at two of the three radiation dose levels. It is pertinent to point out that the mice used in the second X- and γ -radiation experiments conducted in Building 202 were from the same two shipments, were injected on the same day with endotoxin from the same stock suspension, and were housed in the same animal room. The difference in effectiveness of *Pseudomonas* endotoxin in X- and γ -irradiated mice is difficult to reconcile with the many similarities in action of these two radiations and the fact that therapeutic measures are generally equally effective for these radiations. Other experiments are being conducted to clarify this.

TABLE 39

The comparative effectiveness of pyrogens in reducing 30-day mortality in CF No. 1 mice irradiated with X-rays, γ -rays or fission neutrons

Treatment 24 hr before irradiation	Type and dose of radiation					
	X-ray, 700 r, 9 r/min		γ -ray, 1100 r, 7-9 r/min		Fission neutrons, 350 r, 7-9 r/min	
	No. mice	Mortality, %	No. mice	Mortality, %	No. mice	Mortality, %
PP,* 2 μ g	32	62	24	100	31	96
PP, 5 μ g	32	43	-	-	8	100
PP, 50 μ g	76	11	20	75	24	100
TAB	48	14	34	58	38	100
Irradiated controls - no treatment	52	94	24	95	50	100

**Pseudomonas* polysaccharide

TABLE 40

The effect of *Pseudomonas* endotoxin on radiation mortality at various doses of X- or γ -radiation

Dose r	X-ray				Dose r	γ -ray			
	50 μ g PP*		Controls			50 μ g PP*		Controls	
	No. mice	30-day mortality, %	No. mice	30-day mortality, %		No. mice	30-day mortality, %	No. mice	30-day mortality, %
600	23	4	55	43	900	29	65	21	23
650	24	0	56	69	1000	29	65	23	39
700	76	11	52	94	1100	30	70	18	77
750	24	45	53	98					
800	24	37	16	100					

**Pseudomonas* polysaccharide. All mice injected intravenously 24 hr before irradiation.

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X-IRRADIATION OF THE GIANT AMOEBA, PELOMYXA ILLINOISSENSISIII. Electron Microscopy of Centrifuged Organisms

E. W. Daniels and L. E. Roth

Protoplasm from nonirradiated amoebae prevents death when transplanted into supralethally irradiated amoebae.⁽¹⁾ In these fused systems both irradiated and nonirradiated nuclei undergo synchronous mitosis,⁽²⁾ indicating that nuclear damage in the supralethally irradiated cells has been repaired. If the protoplasm within the nonirradiated donor amoebae is stratified by gravitational force and immediately thereafter separated by cutting the amoeba into halves or thirds, the protective capacity resides primarily in the centrifugal portions.⁽³⁻⁵⁾ These contain nuclei, mitochondria, food vacuoles, crystals, and a complex ground substance. After the nuclei are removed from the centripetal halves and thirds of amoebae by $700 \times g$ ⁽²⁾ these fractions retain radiorestorative capacity until smaller cytoplasmic components have also been removed at higher forces. Although transplanted nonirradiated nuclei alone probably have the capacity to prevent death in lethally irradiated Pelomyxa illinoisensis [as in Amoeba proteus^(6,7)] the evidence at present indicates that some components of the cytoplasm also contain radiorestorative capacity.

In the work presented here the contents of the different strata of centrifuged amoebae are examined in detail, with a view toward better understanding the components required for recovery by the supralethally irradiated cells.

About 40 organisms from the Illinois stock of Pelomyxa illinoisensis^(8,9) were subjected to the centrifugation procedure previously described,⁽⁴⁾ and studied with the electron microscope. In the present work four centrifugal force levels were studied: 1000, 3000, 5000 and $7000 \times g$. The time of centrifugation, exclusive of acceleration and deceleration, was 10 min. The stratified amoebae were immediately removed from the tube with a pipette, put into cool, 1% osmium tetroxide and prepared for the electron microscope in the conventional manner. The sections were observed in an RCA EMU-3E electron microscope operated at 100 kv.

For orientation purposes the stratified amoebae were arbitrarily divided into four major zones bounded by structures which stratify out in a more or less constant manner. These zones are not of equal dimensions (Figure 62); on the average, the two nearest the centrifugal pole together contain about one-third of the total protoplasm of the cell, whereas the remaining two each contain approximately one-third. These four zones were lettered from A at the centripetal pole to D at the centrifugal (Figure 62).

Figure 62. Light micrograph of unstained amoeba centrifuged at $7000 \times g$, showing the major zones A, B, C and D. The centrifugal pole is toward the bottom of the page.

Furthermore, as the discussion requires it, each of these four zones can be conveniently subdivided into three parts and designated as centripetal, middle and centrifugal areas. Each of the zones is bounded on the outside by a plasmalemma composed of five layers.

The A zone. This zone is characterized, particularly at the higher gravitational force levels, by closely packed, irregularly outlined vacuolar structures which vary from one to several microns in diameter (Figure 63A). The vacuoles are largest nearest the centripetal end, and they become progressively smaller toward the centrifugal pole. Following forces of $3000 \times g$ or greater, the A zone contains a minimum of extravacuolar fluid cytoplasm, very few if any mitochondria, and occasional large contractile

Figure 63. A. Typical section through zone A after 2000 x g. B. Mid-area of zone B after 7000 x g. C. Lowest portion of B zone, showing the approximately 0.5- μ vesicles after 7000 x g. D. Golgi body found in centrifugal area of B zone. E. Lower B zone showing stratum of Golgi bodies above the supranuclear mitochondrial stratum after 7000 x g. F. Nuclear or C zone after 3000 x g. G. Typical view of the D zone after 3000 x g. The scale, lower right corner of each picture, equals 1 μ .

Symbols used:

C - crystal vacuoles
D - electron-dense bodies
F - food vacuoles
M - mitochondria

N - nuclei
V - vacuole of the B zone type
VI - irregular vacuole
VL - unstained 0.3- μ vesicles

VT - Phosphotungstic acid-stained 0.3- μ vesicles
S - ground substance
GB - Golgi bodies

vacuoles. Evidence for lipid accumulation at the centripetal pole of P. illinoisensis, not shown by electron microscopy, was obtained by fixing and staining the centrifuged amoebae in Sudan III.

The B zone. This is extremely complex, particularly in amoebae centrifuged at the lower gravitational forces; it contains not only large, well stratified components but also a number of different, relatively minute structures which are not well stratified. As the force is increased from 1000 to 7000 x g, progressively better separation is achieved. The most conspicuously stratified components of the B zone are the ground substance, 4- μ vacuoles, Golgi bodies, and supranuclear mitochondria (Figure 62). The bulk of the ground substance of the cell is concentrated in the centripetal half of the B zone but the 4- μ vacuoles (Figure 63B) are largely confined to the middle and centrifugal portions. In the amoebae centrifuged at 7000 x g the Golgi bodies (Figure 63D,E) were confined to the centrifugal half of the B zone immediately above the supranuclear mitochondria. The mitochondria stratified out below the Golgi bodies into the C and D zones. Thus, the more dense mitochondria formed a much wider stratum than the Golgi bodies.

In addition to the four characteristic strata of the B zone that have been mentioned, many smaller components are more uniformly distributed both within the B zone and in the C and D zones. The first of these consists of numerous small (0.1 to 0.5 μ) vesicles of two distinct types (Figure 63C). These appear to be pinocytosis vesicles formed by the invagination of the plasmalemma as it encloses fluid droplets which then become freed in the cytoplasm, and micropinocytosis vesicles which have budded off from the food vacuoles.⁽¹⁰⁾ During centrifugation, both types tend to migrate in the general direction of the centrifugal pole, and even after 3000 x g are seen predominantly in the heavy half of the cell, centrifugal to the middle portion of the B zone. Other minute vesicles ranging from about 100 to 200 m μ appear in considerable abundance in the B and C zones. These appear to have been pushed along in the fluid cytoplasm and are not otherwise stratified by the forces used.

The C zone. With occasional exceptions, the C zone contains all of the nuclei (Figure 63F), even after centrifugation at the lowest force used in these experiments. The C zone contains several particulate components other than nuclei. In all of the amoebae we found distributed throughout the C zone mitochondria and 0.1 to 0.5- μ spheroidal vesicles, with and without internal granules. Large (ca. 0.5 μ) electron-dense particles, encased within vacuoles (Figure 63F), are found in considerable numbers.

The D zone. This contains two components not seen in the other three zones. The first is the crystal vacuole (Figure 63G), of which many are distributed more or less evenly throughout the entire D zone. The line of demarcation between the nuclear zone and the crystal stratum of

the D zone is, almost without exception, quite sharp (Figure 62). The second characteristic component consists of mature food vacuoles (Figure 63G) containing food organisms in various stages of digestion without the culture fluid which surrounds the prey animals immediately after capture.

In addition to food vacuoles and crystals, mitochondria occur in considerable numbers throughout the D zone. This zone also contains the bulk of the approximately $0.5\text{-}\mu$ electron dense bodies which are encased within vesicles. These bodies have not yet been identified, but may be small bacteria or allied forms.

Noncentrifuged amoebae

Nearly all of the structures segregated and stratified in centrifuged amoebae are seen in random relationships in the noncentrifuged organisms. For example, the vacuoles of irregular outline that migrate to the centripetal pole are often seen in close proximity to nuclei, crystals, or mature food vacuoles which, during centrifugation, migrate toward the opposite pole. A section through a contractile vacuole in a noncentrifuged Pelomyxa illinoisensis amoeba showed the usual accumulation of mitochondria around the periphery of the contractile vacuole. Pappas⁽¹¹⁾ and Pappas and Brandt⁽¹²⁾ have clearly shown in Pelomyxa carolinensis and other amoebae that mitochondria, as well as a high concentration of smaller vesicles, accumulate around the contractile vacuoles. The small vesicles apparently fuse and empty into the contractile vacuole which in turn eventually empties into the external medium.

From previous studies⁽²⁻⁵⁾ it is apparent that some components of nonirradiated giant amoebae are not required for recovery and do not have restorative capacity for irradiated cells. These include the structures described in this report which accumulate in the A zone and in the centripetal part of the B zone following centrifugation at $5000 \times g$ or more. Fusion of this portion into supralethally irradiated cells rarely prevents death. On the other hand, the rest of the stratified amoeba has restorative capacity. However, not all of the components in the latter are required for recovery, for although either the middle or the heavy one-third portion of centrifuged amoebae has radiorestorative capacity, each has different as well as common components. It is probable that the essential radiorestorative subcellular unit(s) is among those common to the middle and centrifugal one-third portions of the amoebae, although alternatively one could postulate that more than one type of component, acting differently at the biochemical level, could be restorative. At the present state of investigation the first suggestion, that the radiorestorative unit(s) is among those common to both the middle and centrifugal thirds, seems to be adequate and less complex. We then ask what components are common to these third portions of centrifuged amoebae. The most obvious of these are: nuclei, mitochondria,

pinocytosis vesicles, a small quantity of ground substance, and occasional electron-dense bodies (ca. 0.5μ), which appear to be bacteria or allied organisms within vesicles.

Centripetal halves or thirds of amoebae centrifuged between 700 and $5000 \times g$, are cleared of all nuclei, according to information gained from numerous stained control cells. These portions have the capacity to prevent radiation death, but this is lost at higher forces.⁽²⁻⁵⁾ Therefore, the nuclei are apparently not required for recovery, provided certain other nonirradiated constituents of the cytoplasm are present. In Amoeba proteus the nuclei from nonirradiated cells have radiorestorative capacity.^(6,7)

The large (0.5μ) electron-dense bodies are highly concentrated in the D zone and less so in the B zone; they are mostly eliminated from the A zone by forces of $3000 \times g$ and greater although some remain after $1000 \times g$. In view of the fact that the A zone has radiorestorative value when centrifuged up to some $5000 \times g$ it appears probable that these electron-dense bodies are not required for the prevention of radiation death. The role of these bodies is not known, but, as mentioned earlier, it is our opinion that they represent a cytoplasmic infective organism.

In view of the great difference in the total volume of ground substance in the relatively ineffective A and B zones as opposed to that in the therapeutic C and D zones, it appears unlikely that ground substance is the restorative material. It is conceivable, however, that small amounts of restorative parts of ground substance migrate centrifugally and thus increase survival of recipient irradiated amoebae.

Mitochondria have not been sufficiently separated from either the nuclei or the pinocytosis vesicles to distinguish clearly their separate effect on the recovery of irradiated amoebae. The pinocytosis vesicles of different types described in this paper are in all likelihood the 0.1 - to $0.3\text{-}\mu$ particulates described in earlier work,⁽²⁻⁵⁾ referred to as alpha granules by Mast,⁽¹³⁾ and studied further by Mast and Doyle,⁽¹⁴⁾ who also observed their migration centrifugally in Amoeba proteus.⁽¹⁵⁾ Similar granules were observed in great abundance in Pelomyxa illinoisensis by Kude.⁽⁹⁾ However, the electron microscope has shown the supranuclear mitochondria more distinctly than has the light microscope, partly because of the staining techniques used; consequently it now appears that the middle and centripetal one-third portions of amoebae probably contain more mitochondria than were required previously.^(4,5) As a result of these observations, it is our current opinion that the mitochondria are essential radiorestorative components of living, nonirradiated cytoplasm.

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PROGRESS REPORT: THORIUM REMOVAL

Joan F. Fried and Jack Schubert

Two forms of thorium, monomeric and polymeric, have been compared with respect to their distribution and ease of removal after injection into the rat. In addition, the comparative efficiency of two chelating agents, DTPA and EDTA, has been evaluated for removal of thorium deposited in monomeric form.

Monomeric thorium was injected intravenously as thorium-234 citrate. Deposition occurred mainly in the skeleton, with the soft tissues receiving very little. Treatment, consisting of 4 daily intraperitoneal injections of either CaDTPA (370 mg/kg) or CaEDTA (335 mg/kg), was begun on the second day after thorium injection. The skeletal burden of the DTPA-treated rats was lowered from 60% to 43% of the injected dose with a corresponding reduction in the soft tissues, and the urinary excretion was raised from 12% in the controls to 34% in the treated rats. In contrast, the effect of EDTA treatment was negligible.

Polymeric thorium was injected as a solution of thorium-234 nitrate with added carrier thorium which gave a well-defined Tyndall beam. In this case, the liver and other components of the reticulo-endothelial system comprised the primary site of deposition, and the skeleton received very little. Treatment with CaDTPA, given in the same manner as above, produced no change in the retention. However, when the dosage of CaDTPA was raised to near toxic levels of 660 mg/kg twice daily for 4 days, the liver content was reduced from 53% to 40% of the injected dose, and the urinary excretion was raised from 0.4% in the controls to 11% in the treated rats.

These differences in the behavior of the two forms of an injected hydrolyzable radioelement are readily understandable on the basis of solubility considerations. When such an element is injected as a weak complex, such as citrate, the exchange with OH^- ions and other complexing anions present in the blood is minimized, and the complex is deposited in the tissues either as such or as a thorium-protein complex. A significant percentage of this thorium is available to the solubilizing action of injected chelating agents which have formation constants high enough to overcome the complexing action of the proteins. However, when thorium is injected as an inorganic salt, the marked tendency of thorium to undergo hydrolysis causes most of it to become hydroxylated and polymerized. Deposits of polymerized thorium may be at least partly intracellular in the reticulo-endothelial system through phagocytic uptake and partly

surface in nature. Those which are intracellular are completely unavailable to chelating agents of the polyamino acid type since these are mainly extracellular in distribution. That portion of the polymerized thorium which is extracellular is resistant to the solubilizing action of the chelating agents, but in the sustained presence of high levels of these compounds, a slow depolymerization would be expected to take place.

PROGRESS REPORT: RELATIONSHIP OF PLUTONIUM REMOVAL TO TUMOR INCIDENCE

I. Experimental Design and Preliminary Results

Marcia W. Rosenthal, Joan F. Fried, Arthur Lindenbaum
and Jack Schubert

Removal of a significant amount of plutonium from the body, even when therapy is delayed until after the radioelement has been deposited in the skeleton, is now possible with the use of the chelating agent diethylenetriaminepentaacetic acid (DTPA).^(1,2) An experiment has been initiated with mice to study the influence of delayed therapy with DTPA on the incidence of tumors, especially those of the skeleton. It is designed to correlate the tumor incidence with the degree and pattern of removal of plutonium from tissues and specific histological sites within tissues.

Experimental

A total of 585 CF No. 1 female mice, about 70 days old and having a mean weight of 22.7 g were used, 460 for tumor incidence studies, 125 for an associated study of tissue distribution and excretion of plutonium (Table 41). Mice of the same weight range were assigned at random to each group. Each mouse received a single intravenous injection of $2.6 \mu\text{c/kg}$ of Pu^{239} contained in 0.32 ml of 1% sodium citrate solution. Three days later single daily intraperitoneal injections of 0.2 ml of CaDTPA (500 mg/kg) or saline were initiated. These injections were continued for a maximum of twelve treatments, as shown in Table 41. Mice designated for plutonium analysis were sacrificed at the times shown, and the spleens, livers, and femurs were analyzed for radioactivity as described elsewhere.⁽³⁾ In addition, a liver sample and the femurs obtained from one mouse per group were fixed in neutral formalin for later autoradiographic study. Mice for tumor studies were caged in stainless steel cages with metal screen flooring, with 16 to 18 mice per cage initially. They received water and Wayne Lab-Blox food pellets ad libitum. The mice were weighed regularly and their condition checked daily. When moribund, each mouse was sacrificed with Nembutal and autopsied. Samples of liver, spleen and kidney and of other tissues with grossly visible abnormalities were fixed in 4% neutral formalin preparatory to microscopic study, and, after a roentgenogram was taken, the carcass was similarly fixed.

TABLE 41

Experimental design

Days treated*	Number of mice per group					Time sacrificed after Pu, days
	Animals for tumor incidence study		Animals for plutonium analysis**			
	Pu + Saline	Pu + DTPA	Pu only	Pu + Saline	Pu + DTPA	
0	-	-	6	-	-	2
0	-	-	7	-	-	3
1	-	50	-	7	7	4
2	35	50	-	7	7	5
3	-	50	-	7	7	6
5	-	50	-	7	7	8
8	35	50	-	7	7	11
12	35	105	-	7	7	15
12			-	7	7	91
12			-	7	7	287
Total	105	355	13	56	56	

*One treatment per day, beginning 3 days after injection of plutonium.

**In each group six mice were used for Pu content of bone and liver, the seventh for autoradiographs.

The plutonium injection solution was prepared by adding 3N HNO₃ to a stock of dried plutonium nitrate. After standing for 24 hr the resulting solution was diluted with 2% sodium citrate solution and the pH adjusted with dilute NaOH. The final injection solution was 1% in citrate pH about 6.0, and contained 3.41 $\mu\text{g Pu}^{239}/\text{ml}$. The DTPA solution (0.14 M) was prepared by neutralization of the acid form of DTPA with NaOH, addition of CaCl₂·2H₂O in 1:1 molar ratio of Ca to DTPA, and adjustment of the final pH to 7.2.

The dose of plutonium given (2.6 $\mu\text{c}/\text{kg}$) can be expected to produce bone tumors in approximately 65% of the mice surviving the latent period and to produce multiple tumors in some.⁽⁴⁾ With this high incidence of tumors in the control mice, it should be possible to correlate any changes in incidence with the degree of removal of plutonium. The dose of 500 mg/kg of DTPA was selected because it has been shown to achieve optimal removal of plutonium⁽⁵⁾ and to have no obvious toxicity when repeatedly injected into mice.⁽⁶⁾ An earlier experiment (Experiment 1 in Reference 3) indicated

that 12 successive days of DTPA therapy were enough to achieve optimal removal of plutonium from the bone when initiated 3 days after the radioelement administration. At three days the plutonium content of the blood is less than 1% of the injected dose, and further deposition of significant amounts in the liver and skeleton would not be expected.⁽⁷⁾

Results and Discussion

One year having passed since the injection of the plutonium into the mice of this experiment, an assessment of progress can be made. The radioactivity analysis has been completed and the results reported.⁽³⁾ Briefly, the plutonium content of the bone was reduced from 18.7% to 11.4% of the injected dose and that of the liver from 41.2% to 27.5% after 12 days of DTPA therapy. Shorter periods of therapy had less effect. Analyses of plutonium content of animals sacrificed 91 and 287 days after the plutonium injection indicated that the full 12 days of therapy had removed almost all of the available radioelement from the bone, but suggested that an additional 4% of the dose might have been removed from the liver if a longer course of treatment had been given.

Comparison of these results with those that followed the injection of plutonium in an ionic solution, in conjunction with ultrafiltration studies, shows that a significant fraction of the plutonium in this injection solution was in colloidal form. As a result, a greater proportion of the radioelement was deposited in the liver and spleen with correspondingly less in the skeleton, and the hepatic deposits were much more resistant to removal by DTPA therapy. In terms of tumor incidence, injection of the polymeric solution would, therefore, be expected to produce a somewhat lower incidence of bone tumors and perhaps show a less marked effect of therapy. Plutonium might also be more likely to influence liver and other reticulo-endothelial tissue tumors.

Autoradiographs of femur and liver sections from control and DTPA-treated mice are being prepared to determine the sites of removal of plutonium from these tissues and the effect of tissue location of the radioelement on tumor induction.

Approximately one-fourth of the mice designated for the tumor incidence study had died by one year in both the control and the treated groups (Table 42). The autopsies revealed a fairly high percentage of tumors or enlargements of the reticulo-endothelial tissues in both groups. Since these have not yet been examined microscopically, they will not be reported at this time. The first mouse with a bone tumor visible on the roentgenogram died at 269 days. An interim summary of the bone tumors in mice dying between this date and 365 days has been made, based on roentgenographic examination only (Table 42).

TABLE 42

Bone tumor incidence at 365 days

Treatment		Number of mice		Mice dying between 269* and 365 days			
Compound	Days	Initial	Dead by 365 days	Number of animals	Number with tumors	% with tumors	Number of tumors
Saline	2	34	7	6	1		1
Saline	8	35	8	6	2		5
Saline	12	35	10	8	6		6
Total		104	25	20	9	45	12
DTPA	1	50	15	13	3		4
DTPA	2	49	10	5	0		0
DTPA	3	50	16	12	2		2
DTPA	5	50	13	5	2		3
DTPA	8	50	6	5	1		1
DTPA	12	105	20	15	2		2
Total		354	80	55	10	18	12

*The first mouse developing a bone tumor died 269 days after the plutonium injection.

It includes skeletal growths that had extended outside the normal limits of the bone (positive tumors) and an arbitrary category of probable tumors - areas of obvious skeletal abnormality that in all probability will be found malignant on microscopic examination. A one-to-three ratio of positive to probable tumors was found both in the control and in the treated mice. A consideration of the positive tumors only, or of the summation of both categories as given in the table, indicates that delayed DTPA therapy reduced by over one-half the percentage of mice dying with bone tumors by one year. Only one tumor was found in most of the mice developing bone tumors, whether in the treated or control group. The final tumor incidence at one year, based on microscopic study of the tumors included in Table 42 and of the many additional areas of possible malignancy noted on the X-ray film, will undoubtedly be larger than that reported here. However, insofar as these figures can be considered representative of developing trends, it seems clear that delayed DTPA therapy can reduce the incidence of plutonium-induced bone tumors.

Many have assisted us in the planning and execution of this experiment. We would particularly like to acknowledge the advice of Sylvanus Tyler in planning the experiment, the cooperation of Miriam Finkel in the interpretation of the X-ray films, and the competent assistance of Elizabeth Moretti and William Westfall.

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PROGRESS REPORT: PLUTONIUM REMOVAL

IV. Tissue Distribution of Monomeric and Polymeric Plutonium as Modified by a Chelating Agent

Jack Schubert, Joan F. Fried, Marcia W. Rosenthal
and Arthur Lindenbaum

A previous report⁽¹⁾ demonstrated that the physical state of thorium determines the pattern of its tissue distribution and also the effectiveness with which this pattern can be modified by chelating agents. This report compares the disposition of Pu^{239} and the effectiveness of treatment with the chelating agent diethylenetriaminepentaacetic acid (DTPA) in mice that had received two different preparations of plutonium. Corollary experiments have demonstrated that in one of these preparations the plutonium was in an essentially monomeric or ionic state and almost completely ultrafilterable, and that in the other it was in a colloidal or polymeric form with a large non-ultrafilterable fraction. DTPA is the most effective therapeutic agent yet developed for the removal of plutonium.

Two almost identical experiments were performed: in Experiment 1 the plutonium was in monomeric form and in Experiment 2 it was in polymeric form. The monomeric injection solution was prepared from a stock solution of $\text{Pu}(\text{NO}_3)_4$ stored in 3N HNO_3 . The stock solution was diluted with 2% sodium citrate solution and the pH adjusted with dilute NaOH. The injection solution was 1% in sodium citrate, at pH about 5.5, and contained $8.67 \mu\text{g Pu}^{239}/\text{ml}$, as determined by assay of the alpha activity. The plutonium was $> 90\%$ ultrafilterable. The polymeric plutonium solution for Experiment 2 was prepared from a stock of dried plutonium nitrate. Since this plutonium preparation had remained in the dried state for several years, it contained a large fraction of the insoluble oxide⁽²⁾ which dissolved very slowly in 3N HNO_3 . After standing in the nitric acid for 24 hr the solution was mixed with sodium citrate and the pH adjusted in the manner described under Experiment 1. The injection solution was 1% in citrate, at pH about 6.0, and contained $3.41 \mu\text{g Pu}^{239}/\text{ml}$. However, unlike the solution in Experiment 1, a large fraction, estimated to be one-third of the total plutonium, was non-ultrafilterable. The ultrafiltration technique was one of low pressure filtration through a cellophane membrane as described previously.⁽³⁾ In both experiments, CaDTPA was prepared as a 0.14M solution by neutralization of the acid form of DTPA with NaOH, addition of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in 1:1 molar ratio of Ca to DTPA, and adjustment of the final pH to 7.2.

A total of 140 CF No. 1 female mice, about 70 days old and having a mean weight of 22 g, were used in the two experiments. Those in Experiment 1 received a single injection of plutonium via tail vein of 0.15 ml, or $3.3 \mu\text{c/kg}$, while those in Experiment 2 received 0.32 ml, or $2.6 \mu\text{c/kg}$.

Three days later, single daily intraperitoneal injections of 0.2 ml of either CaDTPA (500 mg/kg, an optimal therapeutic dose) or saline solution were initiated. A total of 18 treatments in Experiment 1, and 12 in Experiment 2 were given. Serial sacrifices were performed by cervical fracture at selected time intervals throughout the experiments. The whole liver and both femurs were removed. In addition, the spleens were removed from mice sacrificed at the time of initiation of the treatment. In both experiments, the livers and femurs from pairs of mice were pooled for plutonium analyses. In Experiment 1, there was one such pair of mice from each group at each time interval, and in Experiment 2 there were three pairs. The spleens were all pooled to make a single sample in each experiment; (3 in the first, and 6 in the second). In Experiment 1, four mice from each group were kept throughout the experiment in a cage equipped for separation of urine and feces, and collections of the excreta from each cage were made regularly.

Tissue and excreta samples were wet-ashed with fuming nitric acid until clear, evaporated to dryness and diluted to 25 ml in 3 N nitric acid. Standards, prepared by the addition of an aliquot of the injection solution to the corresponding tissue or excreta from uninjected mice, were treated in the same manner. Triplicate planchets were prepared from all samples and standards and were counted in an argon gas-flow counter under approximately 50% geometry. The radioactivity of the samples was compared with that of the standards. Skeletal radioactivity was calculated from the femur content by multiplying the mean femur radioactivity by 20.

Results and Discussion

Control Mice. Three days after the plutonium injection the amount accumulated in combined liver and bone was the same in the mice of both experiments, but the partition of the radioelement between these tissues differed markedly. The monomeric plutonium was almost evenly divided between the bone and the liver (Figure 64, lower); whereas in the case of the colloidal or polymeric form, the amount of plutonium in the liver was over twice that in the bone (Figure 65, lower). Further, the percent of the injected dose of the monomeric plutonium in the spleen was 0.1% and of the polymeric plutonium was 1.3%.

Over a period of two to three weeks the plutonium content of bone in the saline control mice of both experiments showed little change, but a striking difference was observed in the liver levels. In mice that received the monomeric form, liver plutonium dropped steadily from 35% of the injected dose at three days to 15% by the end of the third week; in mice injected with the polymeric form it remained almost unchanged at about 44% for the first two weeks. However, a very slow elimination of plutonium

from the liver did take place in the latter experiment since three months later the liver level had dropped to about 21% of the injected dose. Subsequently no further loss took place.

Fig. 64. Retention and cumulative excretion of plutonium following injection of the monomeric form as influenced by daily DTPA therapy.

Figure 65. Removal of plutonium injected in a polymeric (colloidal) form from bone and liver by daily DTPA therapy.

DTPA Treated Mice. DTPA administration to mice that had received monomeric plutonium resulted in rapid removal of the radioelement from the liver (Figure 64, upper); after seven days of treatment the liver burden was reduced to less than 1% of the injected dose. In mice that received the polymeric plutonium, however, DTPA had much less effect on the liver levels; even after 12 days of treatment the mice still had 28% of the injected dose of plutonium in the liver (Figure 65, upper).

The effect of DTPA therapy on the removal of plutonium from the skeleton was similar in both experiments, as shown in the figures. In each case approximately nine consecutive days of treatment removed about half the radioelement from the bone.

DTPA caused a marked increase in urinary excretion of plutonium injected in the monomeric form (Figure 64). Although this increase was greatest after the first treatment, it was still statistically significant after 18 days. Plutonium in the feces also rose following initiation of therapy. However, after 3 days it dropped below the amount found in feces of the control mice, corresponding to the almost complete depletion of the liver burden. The amount of plutonium excreted in the feces was quantitatively related to that lost from the liver in control as well as in treated mice.

The difference in the physical state of the two plutonium injection solutions is clearly reflected both in the tissue distribution pattern of plutonium and in the degree of effectiveness of DTPA therapy. Thus, the colloidal nature of the polymeric plutonium in Experiment 2 is demonstrated by (a) the increased retention of the radioelement in the liver and spleen at the expense of the bone, (b) the relative permanence of its deposition in the liver, and (c) the relative resistance of the hepatic plutonium to DTPA therapy.

Plutonium must be deposited in the liver in at least three forms or fractions, as judged by its rate and degree of elimination and its availability to the chelating agent in these two experiments. One, demonstrated with the polymeric plutonium, is the irreducible amount that remains in the liver apparently indefinitely even with DTPA treatment. This fraction must consist of large particles taken up immediately by phagocytic action into the Kupffer cells. Intracellular plutonium is unavailable to the action of DTPA since cell membranes are relatively impermeable to this agent.⁽⁴⁾ The second form of hepatic plutonium, also demonstrated with the polymeric form, is the fraction that left the liver slowly, over several months in untreated mice, and in about two weeks in treated mice. It is considered to be extracellular and in a polymerized form only gradually solubilized and removed by complexing or chelating action. The third form, comprising essentially all of that deposited in the liver following injection of the monomeric solution, is removed very rapidly by DTPA and is considered to be extracellular in forms which dissociate readily - probably bound to the beta globulin fraction of proteins and as relatively small radiocolloidal particles of plutonium hydroxide.

In the case of the bone, the fraction of available radioelement must have been equally accessible to the action of the chelating agent after injection of either monomeric or polymeric forms since approximately one-half of the skeletal plutonium was removed by DTPA in each case.

We wish to thank Mrs. Elizabeth Moretti and Mr. William Westfall for their competent technical assistance.

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STUDIES ON THE ESTABLISHMENT AND GROWTH OF TUMOR METASTASES USING TRITIATED THYMIDINE

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The establishment and growth of tumor metastases were studied in Cx_A F₁ mice injected via the tail vein with a suspension of Ehrlich ascites tumor cells. Under these circumstances, secondary tumor growths appear almost exclusively in the lungs, with only an occasional secondary growth in the right ventricle of the heart. Tritiated thymidine was used in these animals on the assumption that it is incorporated almost exclusively into DNA prior to mitosis, and, therefore, that cells labeled with tritiated thymidine must be alive and actively proliferating. The incorporation of thymidine into the tumor cells growing in the lungs was followed by autoradiography and by radiochemical measurements with a liquid scintillation counter.

In a first experiment, when a single intraperitoneal injection of tritiated thymidine was given immediately after intravenous injection of tumor cells, it was found that only 8 per thousand of the tumor cells lodging in the lungs were labeled, indicating that the great majority of the injected tumor cells did not survive at the site of arrest. When tritiated thymidine was given 24 hr after intravenous injection of tumor cells, at a time when most of the injected cells had already disintegrated, the percentage of labeled tumor cells was about 15. From the 3rd day on, the uptake of tritiated thymidine measured simply the growth rate of the surviving tumor cells, as, by this time, the non-surviving cells had been eliminated. It was found that, from the 3rd to the 12th day of tumor growth, a single injection of tritiated thymidine gave a constant percentage of labeled tumor cells (about 40).

In a second experiment, repeated injections of tritiated thymidine were given to tumor-bearing animals to determine the fraction of the tumor cell population participating in the proliferating pool. It was found that when repeated injections of tritiated thymidine were given over a span of 20 hr, almost 100% of the tumor cells were labeled, thus indicating that the mean generation time of the Ehrlich ascites tumor cells growing in the lungs was about 20 hr, with almost the totality of the tumor cells actively dividing every 20 hr.

Radiochemical measurements using a liquid scintillation counter followed closely the results obtained by autoradiography but were limited by the heterogeneity of the tissue in which tumor tissue was measured together with fragments of normal lung tissue. It was shown, however, that with homogeneous cell populations, radiochemical measurements could be as accurate, and probably easier to perform, than autoradiographs.

In a third experiment, the number of surviving tumor cells lodging in the lungs, and the growth rate of the survivors, were determined separately in male and female mice. A single intraperitoneal injection of tritiated thymidine was used. The results showed that the number of injected cells surviving at the site of arrest was 10 times larger in female than in male mice. However, once established, the surviving tumor cells grew at the same rate in both sexes. The end result was a higher number of metastases (about 10 times larger) in female than in male mice, all injected with approximately the same number of tumor cells.

The following conclusions were drawn from these experiments:

- 1) only very few of the injected tumor cells survive at the site of arrest;
- 2) some of the surviving tumor cells begin to synthesize DNA within 2 hr after lodging in the lung capillaries;
- 3) after the 3rd day, the tumor cells in the lungs grow at a constant rate with 100% of the cells dividing every 20 hr;
- 4) the number of surviving cells is 10 times higher in female than in male mice;
- 5) in transplantation experiments, it is necessary to distinguish between the "take" and the growth of tumor cells;
- 6) the fact that almost the totality of the tumor cell population participates in the proliferating pool may explain the growth of a tumor, without the need for a faster rate of mitosis;
- 7) these findings indicate that the testing of chemotherapeutic agents should take into consideration the mitotic cycle of a cell population.

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