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DURATION OF LIFE DAILY ^{60}Co GAMMA IRRADIATION: REPORT ON SURVIVAL AND INCIDENCE OF TUMORS

Douglas Grahn, George A. Sacher, John H. Rust,* and R. J. Michael Fry

This report is concerned with some of the analysis of the data obtained from a study of chronic radiation lethality of four strains of mice. The design and the experimental conditions have been previously described⁽¹⁻³⁾ and initial reports on the lethality data and incidence of tumors have been given.^(2, 4-7) Mice at 100 days of age were placed in a γ -radiation facility that automatically irradiated the animals during the night for a period of 7 to 10 hr. The animals were caged in groups of three (except at 0.3 R/day) and irradiated for the duration of life. Both sexes, in equal numbers, of three inbred strains, A/JaxGh, BALB cJGh, and C57BL 6JGh and one hybrid, the BCF₁ (C57BL 6 $\times$ BALB c F₁) were used in the major part of this study. Additional information was obtained on the C57BL 6h strain at doses of 12 R/day and above.

Ten daily-dose levels were employed and are given in Table 1 along with the mean after-survival values. From 12 R/day and above, equal sample sizes were used for all genetic groups; 48 sex and strain at 6 R/day, 12 R/day and 24 R/day; 24 sex and strain at 32 R/day, 43 R/day, and 56 R/day. At 0 R/day through 6 R/day, a double sample was used for the BCF₁; 168 sex at 0 R/day and 1.3 R/day; 96 sex at 2.6 R/day and 6 R/day. The samples were 84 and 48, respectively, for the three inbred strains.

The dose group at 0.3 R/day was an irregular sample. This exposure condition is behind a bulk lead shield in one corner of the γ -radiation facility and does not provide an ideally comparable environment to the balance of the exposure conditions. Caging was also different and involved groups of 12. Originally, only the BCF₁ and BALB c strains were entered owing to space limitation. Sixty mice sex for the inbred and 120 sex for the hybrid were used. Late in the study, the A strain was added as space became available, so there is a degree of discontinuity in time between the 0.3 R/day group in the A strain and the balance of the study. Caging and other microenvironmental

variables induce a general discontinuity for all animals at this dose. This is emphasized because there are certain inconsistencies in the data at this level that cannot be clearly interpreted.

Control mice were held in a maze corridor to the γ room. Generally, the physical environment was comparable to that in the room itself, but there was more ambient temperature variation and greater contact with the general animal facility environment. Here, too, there is some reservation regarding the most rigorous comparative analysis. At any rate, for the dose groups from 1.3 R/day through 56 R/day, the environment was substantially constant.

MORTALITY FROM ALL CAUSES

The survival data are given in Table 1 and Figure 1. In the latter, log MAS (mean after-survival) is plotted against daily dose, and a linear relationship is clearly demonstrated. This was seen earlier by Sacher and Grahn⁽³⁾ and has been substantiated in a half-dozen different genetic strains and hybrids.⁽⁷⁾ The data fit the equation

$$\text{MAS}_D = \text{MAS}_0 e^{-\beta D} \quad (1)$$

The value of β has only varied between -0.033 and -0.043 for different sexes, strains, and hybrids in nearly two dozen separate estimates over the past 10 to 15 years. The simple average for all strains and sexes is -0.038 ; that is -3.8% R/day; -3.8 days/100 R accumulated; $3.8 \text{ MAS}_0 = \% \text{ lost R}; (0.038) \cdot (\text{MAS}_0) = \text{days lost R/day}$.

Because there is no significant genetic variation in this constant, nearly all genetic differences in long-term response are incorporated in the basic differences in life expectancy.

Figure 1 also expands the scale at the lower doses to demonstrate the increase in variation of response. Strain A shows almost no response differential below 6 R/day. Strains BALB c and C57BL show a steady progression down to 0 R, while the BCF₁ demonstrates a small amount of over-survival at 0.3 R/day. This is statistically significant for the males, but not the females.

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TABLE 1. MEAN AFTER-SURVIVAL AND STANDARD ERRORS IN DAYS BY DOSE, SEX, AND STRAIN.
Age at Start of Exposure: 100 Days

Strain	Sex	Daily exposure dose, R/day									
		Control	0.3	1.3	2.6	6	12	24	32	43	56
A/Jax	♂	466 ± 16	377 ± 22	433 ± 16	451 ± 19	458 ± 21	388 ± 15	231 ± 8	182 ± 7	111 ± 4	83 ± 4
	♀	538 ± 16	502 ± 21	528 ± 16	533 ± 20	486 ± 19	400 ± 18	228 ± 7	153 ± 8	102 ± 3	59 ± 1
	♂ + ♀	502 ± 12	436 ± 17	480 ± 12	492 ± 14	472 ± 14	394 ± 11	230 ± 5	167 ± 6	106 ± 3	71 ± 3
BALB/c	♂	569 ± 15	520 ± 28	542 ± 20	461 ± 24	526 ± 16	368 ± 14	234 ± 9	166 ± 7	106 ± 3	60 ± 1
	♀	646 ± 14	643 ± 18	592 ± 16	557 ± 17	473 ± 19	395 ± 10	226 ± 7	158 ± 6	99 ± 4	55 ± 1
	♂ + ♀	609 ± 14	583 ± 17	567 ± 13	509 ± 15	499 ± 13	382 ± 9	230 ± 6	162 ± 5	102 ± 3	57 ± 1
C57BL/6	♂	643 ± 19		572 ± 20	582 ± 24	576 ± 22	468 ± 14	275 ± 10	209 ± 8	136 ± 4	83 ± 1
	♀	647 ± 22		631 ± 16	565 ± 22	581 ± 20	484 ± 14	250 ± 9	179 ± 7	128 ± 3	77 ± 3
	♂ + ♀	645 ± 14	--	602 ± 13	573 ± 16	580 ± 15	476 ± 10	263 ± 7	194 ± 6	132 ± 3	80 ± 2
BCF ₁	♂	710 ± 15	762 ± 18	689 ± 13	698 ± 17	599 ± 12	467 ± 17	301 ± 12	207 ± 8	166 ± 5	99 ± 5
	♀	796 ± 14	815 ± 14	722 ± 12	657 ± 15	580 ± 11	448 ± 12	282 ± 12	212 ± 11	160 ± 5	92 ± 3
	♂ + ♀	753 ± 10	788 ± 11	705 ± 9	677 ± 11	590 ± 8	457 ± 10	291 ± 9	210 ± 6	163 ± 3	95 ± 3

FIG. 1.—Logarithm of mean after-survival (MAS) vs. daily dose of Co⁶⁰ γ radiation.

The basic age-specific death rates for all causes of death, the two sexes combined, are given in Figure 2 for the four genetic groups. The characteristic fan of mortality-rate slopes is evident in all strains though

FIG. 2. Age-specific mortality rates for indicated strains and daily doses. Sexes combined; all causes of death.

the A strain reveals an unchanging array for doses 0 R through 6 R/day. The pathological basis for this apparent lack of radiosensitivity is in the susceptibility of this strain to amyloidosis and kidney degeneration.¹⁵ A careful comparison among the strains indicates that all the A strain low dose groups and the

control have an accelerated mortality rate; the Gompertz slopes for the 0 R through 6 R/day groups are all roughly equal to that at 6 R/day for the BALB c. All radiation injury at doses below 6 R/day in strain A is apparently overwhelmed by the high incidence of degenerative disease. It should be noted that this suppression of response affects nearly all specific end points in the A strain at the low doses, although the response at 12 R/day and above is quite typical of the average mouse.

Figure 3 is a more detailed display of the mortality rates for the BCF₁ for which more adequate sampling was available. The death-rate curves are nearly all substantially nonlinear. This has been discussed previously by Sacher.⁽⁹⁾ It should also be noted here and in Figure 2 that in no case does one find a nonlinear relation that is concave upward as described by Neary⁽¹⁰⁾ for the CBA mouse. The Harwell experience for long-term gamma or neutron irradiation is most peculiar in this regard and is certainly not confirmed by anything we or others have seen.

Some of the nonlinearity of response can be attributed to leukemia, which has a phasic nonlinear increment of its own. Mortality rates derived from the population decremented for leukemia mortality are given in Figure 13 and will be discussed in a later section.

The control data are also given for the full neonatal, infant, and juvenile period. These data are derived from our breeding records for the first 30 days and from stock cage experience for the period 30 to 100 days. Between 200,000 and 1,000,000 mouse-days of experience are now available for the early part of the life table for this hybrid, as well as many of the inbreds. The preweaning death rates tend to be about 10-fold higher than those in human experience in the first 30 days, a common feature in littering animals.

MORTALITY WITH SPECIFIC PATHOLOGY

Autopsies were performed on almost 90% of the mice. Major lesions were grossly defined and about 25 to 50% were studied by microscopic examination. The percentage examined microscopically was highest in the control and low dose groups. Since the same degree of detail was not available for all mice, we decided to analyze the data for lesions for which both the macroscopic and microscopic information gained could be used. Our survey concentrated on:

1. Tumors of the reticular tissue, which were subdivided into two groups, (a) those with thymic involvement and (b) those with no apparent thymic involvement. This arbitrary subdivision was necessary because data obtained by macroscopic examination alone was used. This

FIG. 3.—Age-specific mortality rates for BCF₁ hybrid; sexes combined, all causes of death.

subdivision results in an underestimate of tumors of extrathymic origin which occur late in the life-span.

2. Ovarian tumors and cysts.
3. Pulmonary tumors (primary).
4. Hepatomas.
5. Total tumor incidence, which also included miscellaneous tumors, such as adrenal, skin and connective tissue, mammary gland, and hemangiomas.
6. Renal lesions, often detected by cystic degeneration.

The final percent incidence for the major tumor categories is seen in Figure 4. We wish to emphasize the extreme nonlinearity of the data so as to *discourage* the casual reader and experimenter from attempting to draw grand conclusions about dose-response relationships from such simple expressions of data.

All tumors do respond to low dose; the rather extreme response for ovarian tumors at 1.3 R/day is quite significant. The nearly exponential drop in incidence above 1.3 R/day is apparently a function of two major opposing components—induction by radiation but with little dose-response sensitivity (more of an all-or-none type response; see Figure 9) opposed by progressive life shortening and truncation of the distribution period of expression.

Pulmonary tumors and hepatomas present a similar picture of a rise at the 2 or 3 lower dose levels, then a steady decline as life-shortening effects predominate. Reticular tissue tumors have a biphasic response; the first rise at 1.3 R/day is significant by a Chi-square test ($P < 0.002$) and generally occurs in both leukemia subtypes and in both sexes of all four

FIG. 4.—Cumulative incidence for reticular tissue tumors, hepatomas, and ovarian tumors for strains A/Jax, BALB/c, C57BL/6 and the BCF₁ hybrid combined. Data for pulmonary tumors on A, BALB and BCF₁ only. Sexes combined.

FIG. 5.—Age-specific mortality rates for reticular tissue tumors for combined sexes and strains A/Jax, BALB/c, C57BL/6 and BCF₁.

strains. This is followed by a small drop-back and then a steady rise through 12 R/day. Thymic tumors make up only one-fourth to one-third of the total until 12 R/day, when these suddenly shift to about 70%

FIG. 6. Age-specific mortality rates for reticular tissue tumors, sexes combined, for indicated strains and doses.

and thereafter produce the second phasic response at 24 R and 32 R/day.

Reticular Tissue Tumors

Figure 5 presents the death-rate data for all strains and sexes combined. At the lowest doses the response is linear, and the death rates are generally displaced upward and parallel to the control. This may be a kind of balance between the early-life thymic tumors and the later increment from those not involving the thymus. At 6 R/day the thymic component is beginning to have its effect, and at 12 R/day and above the sharp, but phasic, mortality increment occurs from tumors involving the thymus. At the 6 R, 12 R, and 24 R/day levels, there is a definite return to or near the underlying linear trend. The latter is commonly seen after single doses.⁽¹¹⁻¹⁴⁾

The data for each strain are shown in Figure 6 and while the same general pattern is seen in all, there are some exceptions. Data from the BCF₁ are much like those for all strains (this will be generally noted because they make up 40% of the total sample in the pooled data), and the data at 0.3 R show no response above control. The C57BL shows mostly a thymic tumor response component. The opposite is seen in the BALB/c. Here the nonthymic component is predomi-

ant in response at 0.3 R through 6 R/day. Although a definite thymic tumor response occurs at the upper dose levels, it is more linear and broadly distributed in time. The A strain shows almost no response at 0 R through 2.6 R/day. A sharp thymic tumor response does ultimately emerge at 12 R/day. As will be noted later, the total reticular tissue tumor response at the four lowest doses is apparently not independent of the pathology defined by degenerative renal lesions.

A detailed analysis of the nonthymic tumors in the BCF₁ at the 5 lower dose levels reveals a family of linearly increasing death-rate slopes (Figure 7). These originate from an apparent common intercept at about 400 days of age. Although 0.3 R/day has no apparent influence on this type of leukemia, a significant increase in death-rate slope is seen at 1.3 R, 2.6 R, and 6 R/day. The only comparable bit of information is Upton's report on nonmyeloid, nonthymic tumors of the RF mouse after single doses of X rays.⁽¹⁴⁾ In that instance, an upward, parallel displacement occurred, which would be expected after single doses, in contrast to the present data that show a progressive slope rotation and a common intercept.

FIG. 7.—Age-specific mortality rates for nonthymic reticular tissue tumors in BCF₁ mouse, sexes combined. Data fitted by least squares linear regressions; slopes are: 0 R, 0.0058 ± 0.0005 ; 0.3 R, 0.0064 ± 0.0005 ; 1.3 R, 0.0072 ± 0.0010 ; 2.6 R, 0.0074 ± 0.007 ; 6 R, 0.0127 ± 0.0013 .

FIG. 8. Age specific mortality rates for primary pulmonary tumors; sexes and strains combined for A Jax, BALB/c and BCF₁.

Pulmonary Tumors

The combined data for strains A, BALB/c, and BCF₁ are presented in Figure 8. This tumor provides a good array of responses: none at 0.3 R, some progressive divergence of mortality rate at 1.3 R and 2.6 R, and both divergence and displacement at 6 R and 12 R/day.

The three contributing strains are not remarkable. The A strain shows the suppressed response through 6 R/day noted for other end points. The BALB/c and BCF₁ are individually similar to the combined response. The response in the C57BL/6 was generally below 3 to 5%.

Ovarian Tumors and Cysts

Figure 9 presents the combined data for the four strains. The characteristic to be noted here is in the similarity of response at 1.3 R, 2.6 R, and 6 R/day. The lack of dose responsiveness and the minimal divergence through 600 days of age suggests that this tumor requires a long development period and that induction may require a comparatively low threshold of injury to be sustained. Because the female mouse

is particularly sensitive to radiation-induced sterility,⁽¹⁵⁾ the tumor response may rise rather abruptly to be a potential maximum when sterility is induced. Death rates could then be the same for all doses, and yields would be a direct function of total time at risk beyond about 500 days of age.

The individual strains show some interesting responses (Figure 10). The incidence in the A strain, again, is suppressed by degenerative disease mortality while the C57BL shows a tight cluster of death rates at all doses above control. The BALB/c and BCF₁ indicate some minor degree of dose response, though this is still small compared to the basic difference between irradiated and unirradiated. The 0.3 R/day group of the BCF₁ does show an intermediate position, but the total dose is only several hundred Rads even after two years' exposure. The general conclusions noted from these data confirm many of the observations made on the LAF₁ by Lorenz at doses below 8 R/day.⁽¹⁶⁾

Hepatomas

The data for this tumor are quite meagre, and individual strains never yield more than 14% incidence. The death rates are progressively divergent, linear,

FIG. 9.—Age-specific mortality rates for ovarian tumors and cysts; A/Jax, BALB/c, C57BL/6 and BCF₁ combined

FIG. 10.—Age specific mortality rates for ovarian tumors and cysts for indicated strains and daily dose groups.

and rise from a common intercept of 2×10^{-5} at 500 days of age. To study the dose response of the incidence of hepatomas, the strain of choice is C3H₁, which will give yields up to 60%.⁽¹⁷⁾

Miscellaneous Tumors

The incidence of other types of tumors was low. In general, the results on tumor incidence reported here are in substantial agreement with those given by Lorenz et al.⁽¹⁶⁾ for the LAF₁ exposed daily to doses of 0.11, 1.1, 2.2, 4.4, and 8.8 R/day. Leukemias, pulmonary tumors, hepatomas, and ovarian tumors all demonstrated qualitatively and quantitatively similar levels of response. Unfortunately, earlier studies of our own on the LAF₁ mouse could not fully corroborate those data, as our previous studies did not go below 5 R/day, and it is obvious that much of the valuable tumor information lies at doses below that level. The present data at higher doses are all consistent with our previous reports.⁽¹⁸⁾

Renal Lesions

A form of renal disease often grossly characterized by cystic degeneration and microscopically by amyloid infiltration is commonly observed in mice and

FIG. 11.—Age-specific mortality rates for A/Jax and BCF₁ control populations for indicated causes.

was a common lesion, particularly, in the A and C57BL strains. It reaches a total incidence of 30 to 50% in the A strain, 15 to 35% in C57BL, and 5 to 15% in the BCF₁. In the irradiated groups the highest incidence was in strain A, 60% at 0.3 R day, which accompanied a disproportionately reduced life expectancy (Table 1 and Figure 1).

Figure 11 gives a comparative analysis of death rates for the controls of strains A and BCF₁, for all causes, all tumors and renal lesions. The picture presented by strain A is seen at all the doses below 12 R day with minor exceptions. Deaths from renal lesions overwhelm all other causes and would seem to force the mortality rate upward. At 6 R day, enough early leukemia occurs to emerge from the renal lesion background for a few hundred days between 150 and 450 days of age. In a sense, the renal lesions act as a culling force in strain A that produces a perfect set of cancer morbidity data. Because the death rate slope for renal lesions is constant between 0 R and 6 R day, a redundant series of morbidity data are produced and no information emerges on differential tumor response.

The BCF₁, on the other hand, exhibits no obvious impairment due to the renal lesion. Death rates from all tumors closely follow the death rates from all causes. This is generally seen in the other strains, though the C57BL is somewhat intermediate in its re-

FIG. 12.—Final incidence of all reticular tissue tumors vs. final incidence of renal lesions for dose groups 0 R, 1.3 R, 2.6 R, and 6 R/day. Data fitted by least squares; slopes are: A/Jax, -0.384 ± 0.170 ; C57BL, -0.780 ± 0.177 ; BCF₁, -1.573 ± 0.556 . The combined data were fitted by the equation $y = a + bx + cx^2$; $a = 36.98 \pm 2.94$; $b = -1.18 \pm 0.27$; $c = 0.010 \pm 0.005$.

lations between renal lesion and all-tumor death rates.

The extent of interaction between renal lesions and neoplasia is seen in Figure 12. The final incidence of renal lesions and leukemias for doses 0 R, 1.3 R, 2.6 R, and 6 R day for the three indicated strains show an individual and a combined negative correlation between tumor incidence and kidney lesion. It would thus appear that the systemic or basic metabolic events that underly the renal degeneration also act upon reticular tissue tumors. At the four dose groups employed in this relationship, there is no major dose response for either lesion; the second degree polynomial that describes the data is thus not an indirect function of radiation level.

Curiously, no particular relation exists between renal lesions and the all-tumor category or ovarian tumors. One is tempted to suggest that this degenerative disease has an autoimmune etiology that also influences the hematopoietic system.

Life Shortening Risk Estimates

A detailed application of portions of these data to the general question of estimating the lifetime risk of low dose radiation exposure has been given by Grahn.⁽⁶⁾ One particularly attractive result was derived from the analysis of all causes of death after the removal of all leukemia mortality. This process of decrementing a population for leukemia deaths was carried out on the BCF₁ hybrids, with sexes combined. The resulting age-specific death rate data were fitted by first and second degree polynomials and a

FIG. 13.—Age-specific mortality rates for leukemia decremented populations fitted by first-degree equations forced through common 100-day intercept of 13.5×10^{-6} . BCF_1 , sexes combined. See Table 2.

TABLE 2. LINEAR MORTALITY-RATE REGRESSION EQUATIONS FOR THE BCF_1 ; SEXES COMBINED, LEUKEMIA-DECREMENTED, CONSTANT 100-DAY INTERCEPT (r_0) OF 13.5×10^{-6} ; $r = 13.5 \times 10^{-6} e^{kt}$

Dose, R day	MAS, days	Mortality-rate slope (k)	
		Prob. of death / mouse day $\pm$ S.E. ($\times 10^{-3}$)	kD/k_0
0	752	6.98 ± 0.21	1.000
0.3	781	7.09 ± 0.18	1.016
1.3	686	7.60 ± 0.20	1.090
2.6	678	8.14 ± 0.18	1.166
6	595	9.57 ± 0.21	1.371
12	466	12.78 ± 0.33	1.831
24	340	18.75 ± 0.87	2.687
32	246	29.40 ± 0.64	4.213
43	156	45.72 ± 0.86	6.552
56	96	78.92 ± 4.39	11.309

common intercept for the starting age, 100 days, was estimated from these equations. The data were then refitted, but now all dose groups were force-fit through the common intercept. The results of this last analysis are shown in Table 2 and Figure 13. A later report will deal in more detail with the problem of estimation of not only life shortening risk but also risk for specific causes of death.

The data on life shortening and effects on tumor incidence in this report confirm some of those previously reported as well as adding some new information. The results and the study of suitable methods of analysis will be of particular value in the planning and execution of the Janus Program.

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LONG TERM EFFECTS OF LOW DOSES OF IRRADIATION ON IMMUNE RESPONSIVENESS OF B6CF₁ Anl[Anl 66] MICE WITH AGE

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PURPOSE AND METHODS

This program will investigate the changes in the immune response with age and the effects of neutron and gamma irradiation upon this response. The primary immune response (19S and 7S) is measured in terms of its latent period, rate of development of antibody-forming cells and the number of antibody-forming cells. These factors will be determined for both sexes of the mouse strain B6CF₁ Anl[Anl 66], which is specific-pathogen free (SPF) and will be used both in the JANUS Program and in studies of bone marrow recovery from neutron and gamma irradiation. This strain is long lived and has an LD_{50/30} after X irradiation of about 980 R.

At each age chosen for the test, 4 male and 4 female mice were given a single intraperitoneal injection of sheep erythrocytes. The mice were then sacrificed at different times up to 14 days after the injection, and their spleens were removed. The cells used to examine the immune response were freed by gentle teasing of the spleens into Eagle's medium. An aliquot of the spleen cell suspension was then dispersed on a blood agar plate, and individual hemolysin-forming cells were detected by the clear plaques (hemolyzed) they formed in the agar. This technique

was adapted from that of Jerne et al.⁽¹⁾ Almost all plaques produced are formed by cells producing the highly hemolytic 19S antibody. To detect red cells that are coated with the poorly hemolytic 7S antibody, goat antimouse gamma globulin is added with the complement, as described by Sterzl and Riha.⁽²⁾ This combination results in hemolysis and the appearance of plaques, where 7S antibody has been adsorbed onto the red cells.

PROGRESS REPORT

Results reported last year⁽³⁾ have been extended, so that now we have a measure of immunological capacity for male and female mice, 360, 540, and 720 days old. The earlier data for mice 6, 21, 28, 56, 100, and 180 days old, have been supplemented by retests of 56-, 100-, and 180-day-old mice, so that they represent 16, 12, and 12 mice, respectively. In addition, three sets of 100-day-old mice were given 25, 75, and 150 R of neutron radiation, respectively. At 200, 300, and 400 days of age, 4 mice from each set were immunized with sheep RBC, and their antibody response was measured.

The results in Figure 14 show that immune respon-

FIG. 14.—The peak number of 19S plaque-forming cells per spleen in male and female B6CF₁ Anl[Anl 66] mice given a single injection of sheep erythrocytes at different ages.

FIG. 15.—The peak number of 7S plaque-forming cells per spleen in male and female B6CF₁ Anl[Anl 66] mice given a single injection of sheep erythrocytes at different ages.

siveness, as measured by the production of putative 19S plaques, reaches a peak between 100 to 180 days. The decline has a half-life of approximately 280 days. Males reach slightly higher peak responsiveness than females, and the rate of decline in the two groups is parallel up to 720 days. There is no significant difference between the sexes in their immunological responsiveness at any specific age. The consistently higher number of plaque-forming cells per spleen of males tested at 5 different ages from peak at 100 days gives a probability of approximately 3% that the two sexes respond equally. There is no detectable difference between the sexes for the number of putative 7S plaques per spleen (Figure 15). At this time, we cannot detect any late effects of any of the three doses of neutron radiation.

CONCLUSION

The number of 19S plaque-forming cells per spleen reaches a higher peak in males than in females. The difference between them remains the same during the

decline in immunological responsiveness to 720 days of age, the oldest mice tested. There is no difference between the sexes for the 7S plaque-forming cells. At this time, no late effects (to 300 days) of 25, 75, or 100 Rads of neutron irradiation have been detected.

These experiments will be extended to include 1095-day-old mice. We also will investigate the long term effects of different doses of neutron irradiation on the antibody responsiveness of mice that are immunologically mature.

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STUDIES ON THE CORNEAL EPITHELIUM

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PURPOSE AND METHODS

We have continued our studies of the cell kinetics of the corneal epithelium. Previously, we discussed some of the reasons for choosing this tissue for cell kinetic and radiation studies.⁽¹⁾ The reasons included the accessibility which allows the cell population to be exposed to drugs, labeled DNA, RNA, and protein precursors, and altered environments in a manner similar to that used for *in vitro* cell systems. This accessibility made possible the comparison of the results for the estimate of DNA synthesis by the percent-mitoses-labeled curve method in relation to the route of labeling. *In vivo* cell kinetic studies are almost all carried out with systemic labeling, but our previous results^(1,2) have raised the questions of whether the reutilization and prolonged availability of the label occurred with parenteral labeling.

The purpose of this report is to show the differences in the percent-labeled mitoses after topical and intraperitoneal labeling. Female CF $\times$ 1 Anl[Anl 66] mice, about 95 days old, were used. Tritiated thymidine (³HTdR) 0.36 Ci mM was applied topically and washed off with a solution containing comparable amounts of thymidine 10 min later; mice were sacrificed at intervals between 2 and 18 hr. In the case of

mice sacrificed between 44 and 108 hr, the ³HTdR was washed off with saline. Squash preparations were made, and the percent of mitoses labeled was determined.

In a third group, label and Colemid were applied topically, and mice were sacrificed hourly for 5 hr, and the labeling index of the total population was determined on sections.

PROGRESS REPORT

The results for the percent labeled mitoses are shown in Table 3 and Figure 16. The values for 42, 48, 55, and 60 hr were reported separately last year. These time intervals were done first, as it was clear that these times were most germane to the question of ³HTdR reutilization. The results for the later time intervals confirm and extend the conclusion that the differences in results for the estimates of cell cycle times are dependent on the route of labeling. Perhaps of more interest are the differences in the first part of the curve. The data points for the intraperitoneal group are not shown, as they were reported previously;⁽³⁾ the curve has been plotted using the mean values for percent mitoses labeled at the various times after labeling. The time elapsed between the 50% points on the first part of the curves or the areas under the curves gives estimates of about 14.5

TABLE 3. THE PERCENT MITOSES LABELED AT INTERVALS AFTER TOPICAL APPLICATION OF $^3\text{HTdR}$

Hours after labeling	Percent mitoses labeled, Mean $\pm$ SD	Number of mice
2	1.1 $\pm$ 0.6	2
3	12.6 $\pm$ 12.5	3
4	73.5 $\pm$ 16.0	3
5	83.9 $\pm$ 0.9	2
6	97.4 $\pm$ 4.2	3
8	99.8 $\pm$ 0.4	3
10	99.8 $\pm$ 0.4	3
12	92.6 $\pm$ 6.3	6
14	65.3 $\pm$ 27.4	3
16	20.3 $\pm$ 11.3	3
18	2.3 $\pm$ 2.7	3
44	0.4 $\pm$ 0.6	2
55	1.1 $\pm$ 1.3	2
60	1.5 $\pm$ 0.7	2
68	7.8 $\pm$ 3.1	3
74	2.3 $\pm$ 1.0	3
84	15.3 $\pm$ 9.0	3
96	9.6 $\pm$ 0.4	3
108	14.9 $\pm$ 6.1	3

FIG. 16.—Percent mitoses labeled in corneal epithelium following topical or intraperitoneal administration of $^3\text{HTdR}$.TABLE 4. LABELING INDEX OF CORNEAL EPITHELIUM (TOTAL POPULATION) FOLLOWING $^3\text{HTdR}$ AND COLCEMID TOPICALLY

Time after label, hr	Labeling index $\pm$ S.E.
1	3.4 $\pm$ 0.2
2	3.2 $\pm$ 0.4
3	3.0 $\pm$ 0.5
4	2.7 $\pm$ 0.4
5	2.8 $\pm$ 0.3

and 11 hr for the duration of DNA synthesis for the intraperitoneal- and topical-labeling groups, respectively. Table 4 shows the results for labeling index after topical application of $^3\text{HTdR}$ and Colcemid. Colcemid prevents an increase in the labeling index due to cell division. No increase in labeling index occurred over the period of the experiment, which suggests that the availability of the label with topical administration was less than one hr.

CONCLUSION

The results suggest that topical application can provide a satisfactory pulse labeling method to study cell kinetics in the cornea. It appears that the estimate of the duration DNA synthesis with intraperitoneal injection is an overestimate due, at least in part, to the duration of the pulse of labeling. These results illustrate the importance of determining the duration of the availability of the label in *in vivo* experiments.

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SOME FACTORS THAT INFLUENCE THE UPTAKE OF TRITIATED THYMIDINE. I.

R. J. Michael Fry, Walter E. Kisilewski, Allan B. Reiskin, Anthony R. Salles, and Charlotte L. Weber

PURPOSE AND METHODS

The findings reported last year⁽¹⁾ on the radioactivity in various tissues of two species of hamster following injection of different doses of tritiated thymidine ($^3\text{HTdR}$) of three specific activities raised a number of questions. It was clear that the labeling

index of the corneal epithelium of the Chinese hamster increased between 1 and 3 hr following intraperitoneal injection. It was also found that the total radioactivity in the intestine rose in the interval between 1 and 3 hr. These findings stimulated a re-examination of the factors influencing the duration of availability of

$^3\text{HTdR}$ in *in vivo* experiments. The gut and eye have again been chosen as the tissues for study, but the mouse was used instead of the hamster because of the difficulties in performing intravenous injections in the hamster. Three questions have been asked. (1) Is the uptake of tritiated thymidine ($^3\text{HTdR}$) into crypt cells of the jejunum influenced by the route of injection? (2) Does the uptake last only for a brief period (usually assumed to be about $\frac{1}{2}$ hr)? (3) Is the uptake of $^3\text{HTdR}$ by the gut affected by time of day independent of route of labeling? Male BCF₁ [Anl/Anl 66] mice, 120 days of age, 30-g average weight, were used. The following experiments were done:

1. $^3\text{HTdR}$, 0.5 $\mu\text{Ci/g}$, 0.36 Ci/mM , (this dose and specific activity was used in all the experiments) was injected either intraperitoneally or intravenously at 1200 and 2400 hr, and mice were sacrificed one hour later. Samples of the jejunum and the eyes were removed, and the ^3H content was determined by a liquid scintillation counting method.⁽¹⁾

2. Mice were injected as in Experiment 1 at 1400 hr and sacrificed one hour later. Squash preparations of jejunal crypts were made, and autoradiographs were prepared using Ilford K2 liquid emulsion and an exposure of 4 days. The mean grain count per crypt cell was determined.

3. Three types of determinations were carried out: (a) the number of labeled cells per crypt squash and the ^3H content 3 hr after intraperitoneal and intravenous injection of $^3\text{HTdR}$, (b) the number of labeled cells at 1 hr after intraperitoneal injection of $^3\text{HTdR}$ and 6 hr after $^3\text{HTdR}$ and Colcemid 5 $\mu\text{g/g}$ at 0 and 3 hr, and (c) the number of labeled Colcemid blocked-metaphase cells at 1, 2, 3, 4, and 6 hr after intraperitoneal injection of $^3\text{HTdR}$ and Colcemid. In experiment (a) the number of labeled cells was the sum of cells in *S* and progeny of initially labeled cells. In Experiment (b) if the Colcemid block was satis-

TABLE 5. RADIOACTIVITY IN GUT AND EYE ONE HOUR AFTER INJECTION OF 0.5 $\mu\text{Ci/g}$ $^3\text{HTdR}$, 0.36 Ci/mM

Route	Radioactivity, $\mu\text{Ci} \times 10^{-3}$ mg dry wt (Mean and S.D.)			
	1200 hr		2400 hr	
	Jejunum	Eye	Jejunum	Eye
Intraperitoneal	(^a)	(^a)	7.32 ± 2.7 $n = 3$	0.218 ± 0.04 $n = 3$
Intravenous	5.75 ± 0.6 $n = 4$	0.293 ± 0.07 $n = 4$	5.46 ± 1.1 $n = 3$	0.247 ± 0.04 $n = 3$

(^a) 3 mice were injected IP; in two mice the values were very low, suggesting that the label was not injected correctly.

TABLE 6. NUMBER OF LABELED CELLS AND THE GRAIN COUNT ONE HOUR AFTER $^3\text{HTdR}$, 0.5 $\mu\text{Ci/g}$, 0.36 Ci/mM

	Mean No. of labeled cells/crypt squash $\pm$ S.E.	Mean grain count/crypt cell $\pm$ S.E.
Intravenous	19.7 ± 1.7 (3 mice, 66 crypts)	$10.5 \pm 0.16^{(a)}$ (3 mice, 1512 cells)
Intraperitoneal	59.7 ± 1.7 (3 mice, 71 crypts)	$12.9 \pm 0.2^{(a)}$ (3 mice, 1340 cells)

(^a) The mean grain counts of crypt cells from the two groups were different ($P > 0.001$).

factory, any increase in labeled cells over the 6-hr period would be due to flow of cells into *S* during the 1-to-6-hr period. In Experiment (c) the following assumptions were made: (1) Colcemid does not alter the age distribution, e.g., by altering flux into *S*, and a steady state existed, (2) the metaphase blocking action of Colcemid was complete within a period equal to the minimum G_2 plus *M* period and lasted 3 hr, (3) blocked labeled metaphases did not disappear in 3 hr. If the latter two assumptions were not valid the number of labeled metaphases would be underestimated by this method.

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The results of Experiment 1 are shown in Table 5 and can be summarized as follows. There is no significant difference in the ^3H content of the samples of jejunum and the eye injected at noon and midnight when the label is injected intravenously. Due to the large variation in the values obtained following IP injection at 1200 hr (perhaps due to incorrect injection in two mice) the higher value of ^3H content in the intestine at 2400 hr with IP injection was not significantly different from the value for the IV group. However, these results raise the question whether reported diurnal variations determined by labeling experiments were influenced by the route of injection.

The results of Experiment 2 are shown in Table 6 and Figure 17. Both the number of labeled crypt cells and the mean number of grains per crypt cell were significantly higher following intraperitoneal injection. These results could be due to a pulse of longer duration with the intraperitoneal injection, with or without local uptake from the peritoneal cavity occurring apart from uptake by systemic distribution. Skougaard and Stewart⁽²⁾ previously reported a similar difference in grain counts for crypt cells on sectioned material following intraperitoneal and intramuscular injections. The magnitude of the difference in the mean number of labeled cells per crypt squash

is surprising, considering sacrifice was at one hour after injection. If the grain count distribution in the intravenously injected group resulted in false negatives, then the apparent difference in labeled cells per crypt squash would be increased. These results raise the question of the suitability of intraperitoneal injections for certain types of labeling experiments.

The results for Experiment 3(a) are shown in Table 7. There is an increase in the ^3H content at 3 hr, which is significant at the 0.01 level. The increase in number of labeled cells per crypt squash is not significant.

In Experiment 3(b) the results for the number of labeled cells per crypt squash are shown in Table 7.

FIG. 17.-The frequency distribution of number of grains per crypt cell following (a) intravenous and (b) intraperitoneal injection of $^3\text{HTdR}$, $0.5 \mu\text{Ci/g}$, 0.36 Ci/mM .

TABLE 7. NUMBER OF LABELED CELLS AND ^3H CONTENT OF THE JEJUNUM

	Mean No. of cells/crypt squash	Radioactivity, $\mu\text{Ci} \times 10^{-3}, \text{mg}$
3 hr after $^3\text{HTdR}$, $0.5 \mu\text{Ci/g}$, 0.36 Ci/mM		
Intravenous	62.9 ± 1.4	5.24 ± 0.4 (S.E.) (7 mice)
Intraperitoneal	65.1 ± 2.1	7.73 ± 0.6 (S.E.) (6 mice)
6 hr after $^3\text{HTdR}$ and Colcemid		
Intravenous	42.0 ± 2.6 39.7 ± 1.8 50.6 ± 2.9	
Intraperitoneal	54.3 ± 3.7 53.1 ± 2.8 53.1 ± 2.6

FIG. 18.-The mean number of labeled blocked metaphases per crypt squash as a function of time following injection of $^3\text{HTdR}$ at 0 hr and Colcemid at 0 and 3 hr.

The mean number of labeled cells per crypt squash at 1 hr was 43.4 ± 1.5 (S.E. of the mean) and the 53.5 ± 1.7 at 6 hr; these values are significantly different at the 1% level.

In Experiment 3(c) an estimate of the duration of S was obtained from determining the rate of flow of cells from S to M and the number of cells initially in S . If certain assumptions are made, for example, that a steady state exists, then T , the mean time in S of cells initially labeled N_s may be obtained:

$$T = N_s b$$

since $b = \Delta M/t$, where b is the rate of flow of cells from S to M and ΔM is the increment of labeled blocked metaphases during a period t .

The number of labeled blocked metaphases was plotted as a function of time (Figure 18) and the points were fitted with a regression line. The slope was 7.2 ± 0.6 hr. The mean number of labeled cells per crypt squash was 54.4 ± 1.5 (3 mice, 60 crypts). Substitution of these values gives a value for T of 6.03 hr. This results in about 1 hr less than the value obtained by the percent mitoses labeled methods. It should be pointed out that this method is dependent on the validity of the assumptions and the accuracy, in particular, of the number of initially labeled cells. The results by this method are not affected by protracted availability of label.

DISCUSSION

The importance of the duration of the availability of injected $^3\text{HTdR}$ was recognized and investigated by a number of workers.⁽³⁻⁶⁾ The effect of route of administration on uptake has also been investigated.^(2, 3)

Skougaard and Stewart⁽²⁾ interpreted their results in the following way. If tritiated thymidine is injected intraperitoneally, a fraction of the label may be absorbed directly across the intestinal wall into the intestinal blood supply, resulting in a higher effective dose for the gut compared to other tissues outside the peritoneal cavity. It is of little importance, for in the direct route from peritoneal cavity to crypt cell the intestinal circulation probably plays a minor role, and direct diffusion is more important. The important point is that in some cell kinetic studies the uptake of the label is dependent on the route of injection, and, therefore, the tissue-cell characteristics that one assumes are not the only factors influencing the uptake. Of particular importance to many of the cell kinetic studies is whether the route of injection influences the duration of availability. Time was not a variable in the experiment of Skougaard and Stewart or of Petersen and Baserga.⁽³⁾ Therefore, these studies did not reveal evidence that the duration of availability might differ between intraperitoneal and intravenous administration. A great many of the cell kinetic studies in rodents have been carried out with intraperitoneal injections. Studies on the cell kinetics of ascites tumor cells have been similarly investigated. The results in this report raise the question of the appropriateness of the intraperitoneal injection in any experiment in which duration of availability is of importance. It is also possible that variation of local uptake of label in the gut may make interpretation of diurnal variations in uptake by tissues outside the peritoneal cavity difficult.

CONCLUSIONS

The results of these experiments show that:

1. The uptake of $^3\text{HTdR}$ by crypt cells of the jejunum is greater when this label is given intraperitoneally than intravenously.
2. The duration of availability may be longer when $^3\text{HTdR}$ is given intraperitoneally. Whether the estimate of the duration of DNA synthesis by the percent mitoses labeled method is influenced by the route of injection is being investigated.
3. When $^3\text{HTdR}$ was given intravenously, there was no difference in the total radioactivity in the gut or eye of groups of mice injected at 1200 and 2400 hr. The uptake following injection by the two routes as a function of time of day will be studied further by isolating the DNA and determining specific activities.

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SOME FACTORS THAT INFLUENCE THE UPTAKE OF TRITIATED THYMIDINE. II.

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PURPOSE AND METHODS

Studies dependent on the uptake of tritiated thymidine $^3\text{HTdR}$ in *Rattus natalensis* (Mastomys) have been frustrating because of an inordinate number of apparent failures in labeling. Injections have been given intraperitoneally, partly because of the difficulty in handling these animals for such procedures as intravenous injections. The purpose of this

report is to give the results of an experiment to determine whether anaesthetizing the animals reduced the number of unsatisfactory results in labeling and the interanimal variation in uptake. Two different types of anaesthetics were chosen, (a) ether and (b) Nembutal.

Intraperitoneal injections of $^3\text{HTdR}$, $0.5 \mu\text{Ci/g}$ 0.36 Ci mM , in 0.4 ml saline were given to Mastomys

TABLE 8. UPTAKE OF $^3\text{HTdR}$ IN TISSUES OF CF#1 MICE AND *RATTUS NALAEVENSIS* (MASTOMYS)

Treatment	^3H content, $\mu\text{Ci} \times 10^{-3}$ mg dry wt							
	Duodenum	Jejunum	Ileum	Colon	Testis	Eye	Liver	Skin
CF#1 mice, ether or Nembutal pooled data	6.85 ± 1.0 (S.D.) $n = 5$	7.8 ± 1.0	8.1 ± 2.9	6.7 ± 1.2	1.4 ± 0.3	0.39 ± 0.2		
Mastomys unaesthetized	2.64	3.47	5.99	6.93	0.94	0.34	1.50	0.15
	5.03	5.95	7.08	5.29	2.41	0.38	1.35	0.57
	1.04	0.86	1.13	1.47	0.42	0.221	1.14	0.10
Ether	0.46	0.36	0.21	2.23	0.32	0.21	1.55	0.09
	1.50	1.35	5.18	1.25	0.51	0.21	1.81	0.07
	0.80	0.93	8.31	0.88	0.43	0.25	1.12	0.16
Sodium pentobarbital	0.26	0.31	0.26	10.8	0.21	0.17	0.94	0.04
	2.81	3.90	7.38	6.37	1.41	0.51	2.01	0.51

which were unaesthetized or anaesthetized with (a) ether or (b) sodium pentobarbital (0.4 mg/g). Two hours after injection the animals were sacrificed and samples of 4 regions of the gut, the testis, eye, liver, skin, and other tissues were taken. The tritium content was determined by a scintillation counting method.⁽¹⁾ Results for anaesthetized CF#1 Anl[Anl 66] mice treated similarly are given for comparison.

PROGRESS REPORT

The results are shown in Table 8. It was found that with CF#1 mice neither type of anaesthetic had an apparent effect on the uptake so the results for 5 mice given either ether or nembutal prior to injection have been pooled.

In the Mastomys the ^3H content of tissues showed marked interanimal variation in the three groups. There is no evidence that anaesthetizing the animals improved the reproducibility of results. In fact, though perhaps coincidentally, the *intra*animal variation increased. For example, the regional differences in the uptake of $^3\text{HTdR}$ by the gut of individual animals was very marked in the group given ether. In one animal the ileum had about ten times the amount in other regions of the gut but the levels of ^3H in the other organs suggest that systemic distribution of the label occurred. In one of the animals given Nembutal

the ^3H content in the colon was over 30 times that in the other regions of the gut. In this animal other tissues showed lower levels of ^3H than the average so that the injection may have been into the colon. It is clear that intraperitoneal injections in this species is a very unsatisfactory route for labeling experiments and that anaesthetizing is of no help.

The generally lower ^3H content in the small intestine of the Mastomys compared to that in the CF#1 mice is consistent with the significantly longer turnover times in the jejunum of the Mastomys (about 130 hr).⁽²⁾

CONCLUSIONS

These results show that in the Mastomys the uptake of $^3\text{HTdR}$ following intraperitoneal injection is even more variable than in the mouse.

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SENSITIVITY OF ACATALASEMIC MICE TO RADIATION-INDUCED MOTIVATION

Dale D. Morris and Robert N. Feinstein

PURPOSE AND METHODS

A single exposure of ionizing radiation used to condition an aversion to saccharin-flavored water is well established in animal behavior research.⁽¹⁾ No mechanism responsible for this postirradiation saccharin avoidance behavior has been accepted. A possible explanation is the humoral motivating factor that Hunt, Carroll, and Kimeldorf postulated from behavioral analysis, using parabiont rat pairs with a skin-vascular anastomosis.⁽²⁾ They suggest that this behavioral effect might reflect toxicity from products of cellular breakdown or the loss from the vascular system of an unidentified substance. It also has been postulated that hydrogen peroxide (H_2O_2), produced in irradiated aqueous solutions, may serve as a cue in postirradiation saccharin aversion in mice.⁽³⁾

If radiation-produced H_2O_2 is relevant to humoral mediation of radiation recognition, animals less able to degrade H_2O_2 should be abnormally responsive to postirradiation saccharin avoidance behavior. Such low catalase animals are now available: the "acatalasemic" mouse strain, C_t^b , developed in this laboratory.⁽⁴⁾ This mouse typically exhibits a blood catalase activity 1 to 2% of normal, and catalase activity of its various solid tissues ranges from 8 to 40% of normal.⁽⁵⁾ Acatalasemic mice also are considerably more sensitive to intraperitoneally injected H_2O_2 than are normal mice.⁽⁶⁾ Relatively high survival of the mice after acute whole body X irradiation suggests that their low catalase level may actually protect them from radiation injury.⁽⁶⁾

In the 1968 Annual Report,⁽⁷⁾ we described a test of 20 acatalasemic and 20 normal mice (C_s^a) of the strain from which the acatalasemic mutant was derived. There was no difference between the initial saccharin preference of C_s^a and C_t^b mice. With saccharin as the conditioned stimulus and X rays as the unconditioned stimulus, both strains showed an aversion to saccharin after conditioning. The C_t^b mice exhibited aversion to a lesser extent than did C_s^a mice.

TABLE 9. COMPARISON OF POSTIRRADIATION SACCHARIN PREFERENCE OF ACATALASEMIC (C_t^b) AND NORMAL (C_s^a) MICE AFTER VARYING DOSES OF X RAYS

	Sham	25 R	50 R	100 R
C_s^a	86.8 ^(a)	64.5	17.6	14.4
C_t^b	91.7	98.8	57.8	17.5

^(a) The preference score is the percentage of saccharin consumed of the total liquid consumption.

PROGRESS REPORT

In a second test, postirradiation saccharin preference was compared in C_t^b and C_s^a mice after varying exposures of X rays. Results of this experiment are presented in Table 9. Again, normal mice exhibited a greater aversion to saccharin than did acatalasemic mice. While the greatest difference between these two strains was obtained at an exposure of 50 R ($P < 0.001$), a clear and significant ($P < 0.01$) difference also was obtained at 25 R. Results of this experiment are published in *Nature*.⁽⁸⁾

In later experiments utilizing the saccharin aversion paradigm, we attempted to modify the postirradiation aversion of C_t^b and C_s^a mice by IP and IV injections of catalase (Worthington (CTR, 0.02 ml/g body weight)). Neither route of injection modified the animal's aversion to saccharin in comparison to control animals. Tentatively, we concluded that the catalase molecule may be too large to affect the receptor site involved in the humoral motivating factor when administered in this fashion.

Using a modification of the saccharin aversion paradigm, experiments in which C_t^b and C_s^a mice received an injection of irradiated blood plasma in place of the usual X ray exposure were performed. The blood was drawn from donor C_s^a and C_t^b mice, was irradiated with 30 kR of gamma rays, was centrifuged, and the plasma was injected IP into the experimental C_s^a and C_t^b mice. We were unable to condition statistically reliable aversion to saccharin with this technique. Garcia, Ervin, and Koelling previously had reported saccharin aversion by injecting the serum from animals which had been irradiated before the blood was drawn.⁽⁹⁾ Although further research is necessary for complete specification, the humoral motivating factor may be due to a circulating by-product of energy absorption with toxin-like effects that are humorally mediated, but which requires receptor sites other than humoral sites for generation.

In experiments currently in progress, IP injections of glucose oxidase are administered to C_s^a and C_t^b mice in place of the usual X-ray exposure used in the saccharin aversion paradigm. The dose-response curve for glucose oxidase (which produces H_2O_2 by enzymatic reaction) is similar to that obtained after X irradiation for both groups of animals.

CONCLUSION

The saccharin aversion behavior of normal and acatalasemic mice differs significantly. Because the

only known difference between these two strains is their blood and tissue catalase, hydrogen peroxide must be involved in the aversion phenomenon. If H_2O_2 per se is responsible for the aversion, one would, a priori, expect the acatalasemic strain to be more sensitive, because H_2O_2 will remain intact longer in these animals. This is not the case; the acatalasemic mouse exhibits less radiation-induced aversion. This implies that the actual aversive stimulus is dependent upon the presence of both H_2O_2 and catalase. Because the only products of the catalatic reaction are oxygen and water, neither of which appears a likely candidate, it is suggested that the actual aversive stimulus is a product of the peroxidatic action of catalase; the chemical identity of this product is unknown.

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AN ENZYMATICALLY INACTIVE CATALASE-ANTICATALASE

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PURPOSE AND METHODS

It has been known since 1939^(1,2) that if one species of animal (the rabbit has been most used) is immunized to catalase prepared from liver or blood of another species, the antibodies produced will complex with the relevant antigen to produce a complex which is catalatically active. The presumption is that the primary antigenic site of the catalase molecule is spatially distinct from the enzyme active site.

An acatalasemic and four hypocatalasemic mutant strains of mice have been developed in this laboratory.⁽³⁾ We have recently shown⁽⁴⁾ that the blood catalase of normal, acatalasemic, and hypocatalasemic mice all have apparently identical primary antigenic sites and presumably differ only in the enzyme active site. The possibility has therefore been considered that if one strain of mice is used as blood donor, and another strain as recipient, the recipient mice may generate antibodies which would of necessity be directed to the enzyme active site and hence would produce an enzymatically inactive catalase-anticatalase complex.

In the earlier paper⁽⁴⁾ it was shown that if one strain of mouse is "immunized" against another strain on a schedule satisfactory for the production of rabbit-antimouse blood catalase antibodies, no antibodies are detectable. This is probably not surprising, since

one would expect a priori that *any* other area of the catalase molecule would prove less strongly antigenic than that area which is the primary antigenic site. We have, however, attempted a very much more intensive immunization of mouse strains against each other and have obtained some evidence of the production of antibodies.

Antigens used were saponin-lysed hemolysates of packed but not washed erythrocytes from $C57$ (normal), $C57^a$ (acatalasemic), and $C57^b$ (hypocatalasemic) mice. The exact immunization schedule is not provided in this brief report, but suffice to say that the process of immunization extended over a period of nine months, and was so intensive that fewer than 50% of the mice survived.

Techniques employed for the detection of anti-catalase were standard Ouchterlony double diffusion, and immunoelectrophoresis, both performed with the LKB Produkter apparatus. In the case of mouse-anti-mouse sera, the mere appearance of a precipitin line is considered presumptive evidence of the production of anticatalase, because catalase is considered to be the only molecule with respect to which these strains differ. In the case of rabbit-antimouse sera, however, many precipitin lines are produced, because the immunizing antigen employed was whole hemolysate of mouse erythrocytes. In this case, the catalase-anticatalase line is easily identified by flooding the

TABLE 10. PRESUMED CATALASE ANTICATALASE LINES OBSERVED IN OUCHTERLONY TESTS

Antigen (blood source) ^(*)	Antiserum (immunized mice)	Results
Normal	Normal	Negative
Normal	Acatalasemic	Positive
Normal	Hypocatalasemic	Positive
Acatalasemic	Normal	Positive
Acatalasemic	Hypocatalasemic	Negative
Acatalasemic	Acatalasemic	Negative
Hypocatalasemic	Normal	Positive
Hypocatalasemic	Acatalasemic	Negative

^(*) Antigen is blood lysate from stated mutants or normal (wild type, control) mice. Antiserum is from stated mutant or normal mice immunized with the designated antigen.

slides with H_2O_2 ; ⁽⁵⁾ a flush of oxygen bubbles clearly demonstrates the presence of catalatic activity.

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Table 10 indicates the permutations of antigen donor and recipient used, and precipitin lines (presumed to be catalase-anticatalase) observed. All immunization schedules were identical. It will be observed that normal mice evidently produced antibodies against acatalasemic and hypocatalasemic mouse blood catalase, and acatalasemic and hypocatalasemic mice produced antibodies against normal mouse blood catalase, but acatalasemic and hypocatalasemic mice produced no detectable antibodies against the blood catalase of each other. This is perhaps a predictable finding, because it has been shown⁽⁶⁾ that the differences between the catalases of normal mice and those of all the mutant strains are probably greater than differences between those of mutants.

It should be emphasized that the findings described are not invariable. Even repeat tests of the identical anti-sera sometimes do, and sometimes do not, show precipitin lines. We have, however, seen

clear and unmistakable lines for all those marked as positive in Table 10. We conclude that the mouse-antimouse antibody levels are near the threshold of visual detectability of precipitin lines, and slight, unknown variations in technique make the difference between detection and nondetection.

Although we assume that any mouse-antimouse precipitin line that we see must represent catalase-anticatalase, this is difficult to prove definitively. Flooding of a slide containing mouse-antimouse precipitin lines with H_2O_2 does *not* produce oxygen; this is to be expected if the antibody is directed to the enzyme active site. A more definitive demonstration will have to be devised; we are now in the process of attempting to do so.

CONCLUSION

We have presented presumptive evidence of a catalatically inactive catalase-anticatalase. This was achieved by immunizing normal mice with the blood of catalase-mutant mice, or vice versa. We are attempting to obtain more definitive evidence that the precipitin lines we see are indeed catalase-anticatalase.

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ORGAN AND TISSUE WEIGHTS AND CATALASE ACTIVITIES IN A SPECIES OF WILD DUCK. POSSIBLE RELATIONSHIP TO RADIATION SENSITIVITY

Robert N. Feinstein, Judith B. Howard, and Joann T. Faulhaber

PURPOSE AND METHODS

In last year's report,⁽²⁾ we listed organ and tissue weights and catalase activities in the blue-winged teal (*Anas discors*) and the shoveler (*A. chrypeata*). We intended to include comparable data for the

green-winged teal (*A. crecca*), but at the time of the report we have not yet obtained the birds. They have since been obtained, and the data are now provided.

The original purpose of this work was to attempt to correlate catalase levels of a given species of wild

TABLE 11. TISSUE WEIGHTS AND CATALASE ACTIVITY OF THE GREEN-WINGED TEAL (*Anas crecca*)

Bird	1	2	3	4	5	Average
Weight, g	298	282	321	300	306	301
Sex	M	F	M	M	M	
Liver						
Weight, g	4.77	5.59	5.77	6.25	5.20	5.52
PU/g	198	250	315	148	320	306
Total PU	944	1398	1818	2800	1664	1725
Kidney						
Weight, g	1.88	1.90	1.93	1.99	1.59	1.86
PU/g	104	113	120	160	166	133
Total PU	196	215	232	318	264	245
Blood						
Volume, ml	33.4	28.2	36.0	33.6	34.3	33.1
PU/ml	1.3	1.5	1.5	1.4	1.6	1.5
Total PU	43	42	54	47	55	48
Lung						
Weight, g	3.90	3.50	3.05	4.47	3.37	3.66
PU/g	3.8	5.1	3.3	3.8	3.6	3.9
Total PU	15	18	10	17	12	14
Gizzard						
Weight, g	9.99	8.45	7.37	8.63	7.17	8.32
PU/g	4.3	6.0	3.0	4.5	3.7	4.3
Total PU	43	51	22	39	27	36
Breast muscle						
Weight, g	61.4	57.5	57.6	63.6	64.0	60.8
PU/g	8.9	11.8	11.4	10.3	13.0	11.7
Total PU	546	851	657	655	832	708
Leg muscle						
Weight, g	12.4	10.0	11.1	10.6	12.8	11.4
PU/g	5.3	7.1	6.1	6.0	4.8	5.9
Total PU	66	71	68	64	61	66
Total measured PU	1853	2646	2861	3940	2915	2843
Total PU/g body weight	6.2	9.4	8.9	13.1	9.5	9.4

⁽¹⁾ Total blood volume estimated at 11.2 ml/100 g body weight. PU = perborate units of catalase activity.

duck with the sensitivity of the species to whole body X radiation, and determined by Tester et al.⁽²⁾ This possible correlation was suggested by the fact that we have been able to demonstrate such a relationship in mutant strains of mice.⁽³⁾

Methods are described in the earlier report.⁽¹⁾

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Table 11 lists weight and catalase activity data for *A. crecca*. These may be directly compared with the data of Tables 42 to 44 of the earlier report.⁽¹⁾

Table 12 combines data from the previous report⁽¹⁾ with data from the present report and with data from Tester et al.⁽²⁾ It will be observed that high catalase activity shows a positive correlation with

TABLE 12. COMPARISON OF WHOLE BODY CATALASE LEVELS AND LD₅₀ OF X RADIATION

Species	Total measured catalase, ⁽¹⁾ PU/g	LD ₅₀ of X rays, R
<i>A. platyca</i>	13.1	894
<i>A. discors</i>	10.7	715
<i>A. crecca</i>	9.4	485

⁽¹⁾ Data of center column were obtained by summing total catalase activities of the tissues actually measured (Table 11 of present report and Tables 43 and 44 of earlier report⁽¹⁾), and dividing by total body weight. PU = perborate units of catalase activity. X ray LD₅₀ data are taken from Tester et al.⁽²⁾

TABLE 13. BREAST AND LEG MUSCLE CATALASE OF WILD DUCKS AND DOMESTIC CHICKENS

Species	Catalase activity, PU/g		Catalase ratio, breast/leg	P value
	Breast muscle	Leg muscle		
<i>A. platyca</i>	10.1 ± 2.3	6.6 ± 2.8	1.5	N.S.
<i>A. discors</i>	8.1 ± 2.0	5.2 ± 0.8	1.6	<0.02
<i>A. crecca</i>	11.7 ± 2.3	5.9 ± 0.9	2.0	<0.001
<i>G. domesticus</i>	1.0 ± 0.2	4.2 ± 1.0	0.2	<0.001

Data for the wild ducks are extracted from Table 11 of the present report and from Tables 43 and 44 of the previous report.⁽¹⁾ Chickens were white leghorns, 3 months old, killed by intravenous overdose of Nembutal. P values compare mean catalase activity of breast and leg muscle for the given species. N.S. = not significantly different.

high radiation resistance. This is in contradistinction to the situation observed in mutant mouse strains,⁽³⁾ where high body catalase correlated negatively with high radiation resistance. It should be pointed out, however, that in the case of the ducks, only three groups were involved, so that is difficult to claim statistical significance for any "correlation."

Because of the observation that in all three species of wild duck, the catalase activity of breast muscle is higher than that of leg muscle, the possibility was considered that muscle catalase is in some way related to functional activity. To add information on this point, we assayed the catalase activity of leg and breast muscle of the domestic chicken. The results, together with comparable information for the three species of wild duck, are presented in Table 13, which augments the suggestion that muscle catalase may somehow be related to functional activity.

CONCLUSION

The catalase levels of three species of wild duck correlate positively with the X-ray resistance of the three species. This is in contrast to the negative correlation of the same parameters earlier shown for

mutant strains of mice. Also, whereas the catalase activity is greater in the breast muscle than in the leg muscle in all three species of wild duck, in the case of the domestic chicken, the catalase activity is greater in the leg muscle than in the breast muscle. A relationship of muscle catalase level to functional activity is suggested.

It is a pleasure to acknowledge again the continued cooperation of Mr. Harvey K. Nelson and Dr. Charles W. Dane of the Northern Prairie Wildlife Research Center in providing the green-winged teal and relevant information.

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EFFECT OF WHOLE-BODY X RADIATION ON FLAVOPROTEIN ENZYMES

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PURPOSE AND METHODS

The literature of radiation biochemistry is replete with studies of the effect of whole-body X radiation on tissue enzymes. One class of enzymes, however, which appears to have been largely ignored in this respect is that of the flavoprotein enzymes. At the time of writing, only four papers⁽¹⁻⁴⁾ and one abstract⁽⁵⁾ have appeared touching on this subject. These reports suggest increases in xanthine oxidase and α -hydroxy acid oxidase in liver after whole-body X radiation, while no effect was observed on uricase or L-amino acid oxidase in liver. Doses, times and rodent species differ amongst these various reports.

It therefore appeared to us to be worth while to attempt a systematic survey of the effect of whole-body X radiation at various times after various doses on the activity level of various flavoprotein

enzymes in mouse liver and kidney. Enzymes examined are listed in Table 14 with some details of assay. All assays were done on whole homogenates of tissue. All incubations were at 37°C. In some instances the assay methods were slightly modified from the references cited.

Mice used were male CF $\times$ 1/An[Anl 66] 10 to 14 weeks of age. Assays were done on liver and kidney of unirradiated mice and mice 3 hr, 6 hr, and 1, 2, 4, and 7 days after 5, 50, or 500 R of whole-body X radiation. Radiation was obtained from a machine operating at 250 kVp and 15 mA, with a dose rate of approximately 40 R/min. Ten mice were used for each enzyme assay at each time and dose point, except that if it became evident that a particular enzyme activity was detectable at none of the time-dose points, only 5 mice were used in each instance to establish this fact.

TABLE 14. FLAVOPROTEIN ENZYMES EXAMINED IN NORMAL AND IRRADIATED MICE

Enzyme	Substrate	Ref.	Homogenate concentration, %		Incubation time	
			Liver	Kidney	Liver	Kidney
L-amino acid oxidase	L-leucine	6	8	8	30 min	30 min
D-amino acid oxidase	D-alanine	6	8	2	30 min	30 min
L- α -hydroxy acid oxidase (short chain)	Glycolic acid	6	4	8	30 min	30 min
L- α -hydroxy acid oxidase (long chain)	DL- β phenyl lactic acid	6	8	2	30 min	30 min
Xanthine oxidase ^(a)	Hypoxanthine		2	2	2 hr	2 hr
Uricase ^(b)	Uric acid		1	2	5 min	2 hr
Monoamine oxidase	Kynuramine	7	2	2	15 min	15 min
Diamine oxidase	Putrescine	8	8	8	4 hr	4 hr

^(a) Xanthine oxidase was assayed by following the increase in absorption at 295 nm in 0.1 M borate buffer, pH 8.5, in the presence of the uricase inhibitor 2,8-dihydroxy-6-methylmercaptapurine at 10^{-4} M.

^(b) Uricase was assayed by following the decrease in uric acid absorption at 295 nm in the same buffer.

TABLE 15. SUMMARY OF EFFECTS OF WHOLE BODY X RADIATION ON FLAVOPROTEIN ENZYMES

<i>Class I. No activity detectable at any time</i>	
a.	Liver L-amino acid oxidase
b.	Kidney L-amino acid oxidase
c.	Liver D-amino acid oxidase
d.	Kidney L- α -hydroxy acid oxidase (short chain)
e.	Liver L- α -hydroxy acid oxidase (long chain)
f.	Kidney xanthine oxidase
g.	Kidney uricase
h.	Liver diamine oxidase
i.	Kidney diamine oxidase
<i>Class II. Activity detectable but not modified by irradiation</i>	
a.	Liver L- α -hydroxy acid oxidase (short chain)
b.	Liver xanthine oxidase
c.	Liver uricase (postirradiation increases noted in mean level are not statistically significant)
d.	Kidney monoamine oxidase.
<i>Class III. Postirradiation changes in activity</i>	
a.	Kidney D-amino acid oxidase (increased after most times and doses)
b.	Kidney L- α -hydroxy acid oxidase (long chain) (increased after most times and doses)
c.	Liver monoamine oxidase (early increases after low doses; late decreases after all doses)

At the proper time after a given X-ray dose, mice were sacrificed, and liver and kidneys were removed, frozen in dry ice-acetone, and maintained in liquid nitrogen until assay. In each day's assays, an attempt was made to include tissues from a variety of dose-time areas, rather than doing one whole series of comparable tissues as a group.

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General observations are summarized in Table 15. Complete data are too copious for the present brief report.

CONCLUSIONS

The eight flavoprotein enzymes studied in both the liver and the kidney of mice at various times after varying doses of whole-body X radiation do not behave as a homogeneous group. Four of the enzymes searched for in liver and five searched for in kidney were not detected. Of the enzymes that did display

detectable activity, three in liver and one in kidney were essentially unaffected by any dose of X radiation at any time observed.

Only three enzymes were observed to be affected by whole-body X radiation:

a) The D-amino acid oxidase of kidney exhibited increased activity at most times after most doses. No significant correlation was apparent with either time or dose.

b) The L- α -hydroxy acid oxidase (long chain) of kidney similarly showed increased activity at most times after most doses. Again no significant correlations were observed.

c) Monoamine oxidase activity in liver showed the most interesting and most significant pattern of postirradiation changes. Significant increases were seen early (6 hr) after 5 and after 50 R, and significant decreases were seen later (4 days) after all doses. By 7 days after all doses, activity had returned approximately to normal.

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MICROVASCULATURE AND LOOSE CONNECTIVE TISSUE OF THE CHICK MESENTERY. FREEZE-ETCH OBSERVATIONS

S. Phyllis Stearner and Margaret H. Sanderson

PURPOSE AND METHODS

Ultrastructural changes in the microvasculature of the chick embryo within one to two hours after lethal X irradiation have been reported.⁽¹⁾ A rapidly developing edema accompanied the vascular injury and has been observed in the irradiated chick as well as the chick embryo. These effects suggest that there are significant early alterations in the extracellular ground substance surrounding small blood vessels. Study of the ground substance is difficult, however, because of its low density and solubility in the usual tissue fixatives. Some years ago, the fine structure of ground substance in freeze-dried tissue was described as a system of submicroscopic vacuoles (600 to 1000 Å) of a colloid-poor material surrounded by a denser colloid-rich phase.⁽²⁾ In light microscope studies, structures visible in special preparations are believed to represent clusters ($>1\ \mu$) of such submicroscopic vacuoles.⁽³⁾ The freeze-etch technique^(4, 5) provides a physical method of fixation that preserves the ultrastructure of extracellular as well as intracellular material with a minimum amount of fixation artifact. In addition, a three-dimensional representation of organelles and surface structures is obtained. The chick mesentery was selected for study with this technique because (1) it is similar histologically to the extraembryonic membrane of the chick embryo used in our previous studies and (2) the small mass required for rapid freezing can be obtained without disturbing the relationship of the cells and ground substance. Preliminary observations of the microvasculature and loose connective tissue are reported here. Subsequent investigation involving this technique will include study of the alterations that follow lethal radiation exposure.

Chicks, two to three weeks of age, were lightly anesthetized with ether. Small pieces of mesentery were excised, placed in a glycerine phosphate buffer solution, pH 7.2, and cut into pieces of less than 1 mm². To prevent formation of ice crystals and permit vitrification of tissue water during freezing, these tissues were treated with two glycerine solutions (one hour each, in either 10 and 20%, or 20 and 30% glycerine). The mesentery is extremely thin when spread; therefore, it was allowed to contract to ensure adequate thickness for fracturing after freezing. After glycerine treatment, the specimens were placed on scarified, degreased copper discs (3 mm in diameter),

frozen in Freon 12 (-150°C), and stored in liquid nitrogen (-196°C) until used. The freeze-etch procedure, carried out in a Balzers apparatus, employed a specimen temperature of -120°C , a knife temperature of -150°C , a vacuum at the time of fracturing of 10^{-6} torr, and a temperature at shadowing of -120°C . The fractured surface was shadowed immediately with platinum-carbon at an angle of 45° , followed by a supporting film of carbon at an angle of 90° . The cold knife holder served as a cold trap to minimize contamination of the replica surface until the specimen could be removed from the chamber. Commercial bleach was used to free the replicas from the underlying tissue and to clean them. Replicas were rinsed in several changes of distilled water, mounted on 200- or 300-mesh gold grids and viewed in a JEM-7A electron microscope.

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The fine structure of a small blood vessel and the surrounding loose connective tissue, as observed in ultrathin sections of material taken from a chick, is shown in Figure 19. The endothelium and an adjacent pericyte are surrounded by a well-developed basement lamina, whereas the ground substance, in which collagen fibers and fibroblasts occur, is a pale-staining material with little apparent structure. A small plasma-filled blood vessel, as displayed in a freeze-etch replica, is shown in Figure 20. Collagen

FIGURE LEGEND SYMBOLS

- BL = Basement lamina
- C = Collagen fibers
- E = Endothelium
- F = Fibroblast
- G = Golgi complex
- ICM = Inner cell membrane
- INE = Inner nuclear envelope
- IS = Intercellular space
- J = Intercellular junction
- L = Lumen
- M = Mitochondrion
- N = Nucleus
- NP = Nuclear pores
- OCM = Outer cell membrane
- ONE = Outer nuclear envelope
- P = Pericyte
- PV = Pinocytotic vesicle
- R = Erythrocyte
- V = Vesicle

In each figure, the large arrow indicates the direction of shadowing.

FIG. 19.—Section of Epon-embedded loose connective tissue from a 3-week chick. Glutaraldehyde and osmium fixation. The small blood vessel contains 2 erythrocytes (R). The basement lamina (BL) is seen as an electron dense band surrounding the blood vessel, whereas much of the interstitial space (IS) is occupied by material of low density in which collagen fibers (C) and fibroblasts (F) are present. $\times 10,000$.

FIG. 20. Cross fracture of small blood vessel in the mesentery of a 3-week chick, from a freeze-etch replica. The surrounding interstitial space (IS) contains fibroblasts and collagen fibers (C) in the ground substance. $\times 19,000$.

FIG. 21.—Greater detail of the vessel shown in Figure 20. Many pinocytotic vesicles (PV) are present, but few organelles can be distinguished. $\times 43,000$.

FIG. 22. Fracture surface showing fibroblasts and a small blood vessel from the mesentery of a 3 week chick. Plasma membranes *en face* of several fibroblasts are shown. Cross fracture of a small vessel and adjacent fibroblasts (upper right) reveals a variety of organelles, shown in greater detail in Figures 23 and 24. $\times 10,000$.

FIG. 23 Enlargement of part of endothelium (E) and fibroblast (F) in Figure 22. Surface structure of inner nuclear envelope (INE) reveals several nuclear pores (NP). Irregular appearance of the ground substance which fills interstitial space (IS) suggests a system of vacuoles. $\times 37,500$.

FIG. 24. Golgi region (G) of both endothelial cell and fibroblast includes lamellae and vesicles. Pits in the plasma membrane lead into pinocytotic vesicles. $\times 32,000$

FIG. 25. A: Portion of an erythrocyte in the lumen of a vessel. Inner cell membrane (ICM) has granular surface. Fracture surface of mitochondria (M) reveals cristae. $\times 17,200$. B: Interstitial region contains groups of collagen fibers (C). Some structure is apparent in the surrounding ground substance, but fibers show no periodicity. $\times 37,000$.

fibers and portions of fibroblasts can be distinguished in the perivascular region. The appearance of the plasma is similar to that of the ground substance that fills the interstitial space (Figure 21). There is no evidence of a basement lamina immediately surrounding the endothelium. A larger area of the connective tissue adjacent to a small blood vessel is shown in Figure 22. Surface fractures of several fibroblasts reveal details of extensive areas of both the inner and outer surfaces of the plasma membrane. A part of the endothelium and a fibroblast (upper right) are shown at higher magnification in Figures 23 and 24. The ground substance in the interstitial space has a definite structural pattern that resembles the system of vacuoles (600 to 1000 Å) described by Bondareff⁽²⁾ and by Chase,⁽³⁾ using other methods of tissue preparation. This vacuolated structure can be contrasted with the more granular appearance of the cytoplasmic matrix. A surface fracture of a fibroblast nucleus (Figure 23) demonstrates the two layers of the nuclear envelope and nuclear pores. A Golgi complex, containing both vesicles and lamellae, is present in a fibroblast and also in an endothelial cell (Figure 24). An endothelial cell junction is shown but tight junctions cannot be distinguished. In the attenuated endothelium of a larger vessel (Figure 25a) there are few pinocytotic vesicles. The erythrocyte, shown in part, has a homogeneous, granular cytoplasm and few organelles. At higher magnification the fibrillar structure of collagen fibers can be seen, surrounded by ground substance (Figure 25b).

PROTECTION AGAINST EARLY RADIATION INJURY TO THE MICROVASCULATURE

S. Phyllis Stearns and Emily J. B. Christian

PURPOSE AND METHODS

Vascular injury and terminal circulatory failure have been described in the chick embryo following lethal irradiation.⁽¹⁾ This early damage frequently results in mortality within a few hours, not only in the chick embryo, but in the young chick and the adult rooster as well. Vascular damage has also been demonstrated in a variety of mammalian species and is important in development of tissue damage that follows exposure to ionizing radiations. Radiosensitivity in terms of dose and time during or between exposures indicates that reversal or resistive processes occur during a sufficiently protracted exposure or during the interval between the two fractions of a

CONCLUSION

Three-dimensional replicas obtained by the freeze-etch technique have provided information about the fine structure of small blood vessels and the perivascular loose connective tissue of the mesentery of the young chick. This method is well-suited for the preservation of connective tissue in a more normal state than can be obtained in fixed material and will be used in connection with studies of radiation damage to these structures.

We thank George T. Chubb for invaluable advice and assistance in applying the freeze-etch method to our material, and Betty Jean Wright for helpful information about specimen preparation. We also acknowledge the efficient service of George T. Chubb and Paul W. Ellwanger in maintaining the Electron Microscope Center.

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split exposure to reduce the radiation injury; therefore, the effects leading to vascular degeneration can be reversed or prevented at an early stage. Early radiation injury can also be reduced by pretreatment with the antiprotease, soybean trypsin inhibitor (SBTI).⁽²⁾ Thus we have attempted to analyze early radiation injury mechanisms and the rapid repair or reversal processes by use of procedures or agents that modify the response. Demonstration of radioprotective procedures are not only of practical significance, but also may provide information concerning the nature of radiation injury mechanisms.

In studies of the microcirculation in the living chick embryo described in this report, early radiation

FIG. 26. Microvascular damage within 1 to 2 hr after 1100 R X irradiation. (a) Control network of small vessels in area pellucida ($\times 280$). (b) Detail of control vessel wall 28 hr after explantation ($\times 900$). (c) Occluded vessel; irregular vessel walls. Thrombus occludes upper vessel ($\times 280$). (d) Collapsed vessel; trapped cells partially lysed ($\times 280$). (e) Vessel occluded by thrombus; vessel wall swollen, vacuolated ($\times 900$). (f) Tissue segmental disappearance of small vessels in area pellucida ($\times 44$).

damage was shown to be reduced in embryos pretreated with a 'conditioning' radiation exposure to 700 R of X ray 4 hr before the second or challenging exposure. A similar reduction in damage to the microcirculation was observed in embryos that received preirradiation treatments of soybean trypsin inhibitor.

Observations of the microcirculation in the living chick embryo were made on the area pellucida, the transparent extraembryonic membrane. For microscopic study of the vasculature with transmitted light embryos were explanted onto a solid medium ac-

cording to the method previously described (9). For treatment with soybean trypsin inhibitor, 1.0 mg in 2 ml Ringer's solution was applied to the surface of the explant (ventral aspect of the embryo). After 5 to 10 sec the solution was drained off and the surface rinsed once with 2 ml Ringer's solution. Control explants were rinsed twice with Ringer's solution. The surface of the explant was then covered with mineral oil in the usual manner. Explants were examined microscopically for evidence of injury before being accepted into the experiment. Radiation exposures were to 250 kVp X rays; physical conditions included

1.0 mm aluminum and 0.5 mm copper filtration; target distance and exposure time were varied. Each dose of the split exposures was delivered in 12 min. SBTI-treated embryos received 1100 R, delivered at 95 R/min at 20 to 30 min after application of the drug. Explants were irradiated in a revolving, heated Lucite chamber maintained at a temperature of 38°C. Irradiation was carried out in an atmosphere of 40% O₂, 5% CO₂, and 55% N₂. Embryos were also incubated in this atmosphere after irradiation.

The protective effect of SBTI against early radiation mortality was not tested on 3-day embryos *in ovo*, for at this stage of development specialized techniques for intravenous administration of the drug are required. Application to the ventral surface of

FIG. 27.—Diapedesis and formation of small hemorrhage after lethal radiation exposure (1100 R). (a) Penetration of circulating cell into vessel wall. $\times 900$. (b) Cell trapped between layers of vessel wall. $\times 900$. (c) Group of extravasated blood cells, vessel occluded. $\times 280$.

FIG. 28.—“Protective” effect of a conditioning exposure on radiation injury to the microvasculature. (a) No apparent damage at 1 hr after first exposure (700 R). $\times 44$. (b) At 6 hr after second exposure of 1100 R (10 hr after 700 R), microvasculature appears unchanged. $\times 44$. (c) Normal vessel wall at 24 hr (20 hr after 1100 R). Compare to control after 28 hr in explant (Figure 26b). $\times 900$.

the blastoderm, however, is probably similar in effectiveness to intravenous injection, for the vasculature is readily accessible to a topically applied substance.

RESULTS

The 3-day embryo has a well-developed vascular system; in explant the structural detail of small vessels in the area pellucida can be observed at

FIG. 29 Protective effect of soybean trypsin inhibitor on radiation damage to the microvasculature. (a) Extensive stasis and segmental disappearance of small vessels at 3 hr after 1100 R X irradiation ($\times 7$). (b) No apparent vascular damage at 8 hr after X irradiation (1100 R) in embryo pretreated with SBTI ($\times 13$).

high magnification (Figure 26a and b). The undifferentiated vessel walls consisted of 2 or 3 cell layers. Circulation was maintained in explant for more than 24 hr, and there was continued growth of the embryo; during this time the vessel walls remained thin and sharply outlined.

A lethal radiation exposure (e.g., 1100 R X rays, delivered in 12 min) resulted in rapid degeneration of the microvasculature. Occlusion of individual vessels developed within 1 or 2 hr, and generalized stasis and death usually occurred within 4 to 8 hr. Microscopic changes observed in the living animal have been described previously.⁽¹⁾ Briefly, the major effects included swelling and vacuolization of the vessel wall, occlusion by thrombi, and finally collapse and segmental disappearance of small vessels (Figure 26e-f). Penetration of a circulating cell through the vessel wall and a resulting small hemorrhage are shown in Figure 27. Lysis of the vessel and entrapped cells frequently proceeded rapidly. Mortality within 12 hr exceeded 80%, and less than 5% survived at 24 hr.

Protective Effect of an Initial or Conditioning Exposure on Subsequent Radiosensitivity

An initial or conditioning exposure of the explanted 3-day embryo to a minimal lethal dose of X rays

(700 R) resulted in a transient resistance to a subsequent irradiation. The protective effect was similar in embryos exposed *in vivo* and in explant. Vascular damage was minimal after exposure to 700 R alone, and mortality within 24 hr was less than 5%. Most vessels remained functional, and only in a few instances were there occluded vessels and vacuolated walls. A second exposure of 1100 R delivered after an interval of 4 hr resulted in 40% mortality within 12 hr (compared to more than 80% in embryos exposed to 1100 R only). Effect of a split exposure on the microvasculature is shown in Figure 28. There was little change in the small vessels after the first exposure, and little additional injury followed the second or challenging dose. A few nonfunctioning vessels developed, but these minor changes occurred slowly over many hours, and frequently little additional degeneration was seen even after 24 hr. Many vessel walls remained thin and distinctly outlined (Figure 28e). Some vessels with evidence of injury, e.g., swollen vessel walls and thrombocytes adhering to localized areas, continued to function for the duration of the observation period. Examples of temporary occlusion were observed in other instances, but after a short time the adhering cells became dislodged and circulation was resumed.

Protective Effect of Soybean Trypsin Inhibitor (SBTI)

In explanted embryos pretreated with SBTI, 50% survived at 12 hr after 1100 R X irradiation; of these, most had a strong regular pulse and good circulation at 24 hr. There was little change in the microcirculation; most vessels were functional and appeared normal after 8 hr, compared to extensive vascular damage in unprotected irradiated embryos (Figure 29). In many embryos, the architecture of small vessels remained unchanged at 24 hr. When a few degenerated or occluded vessels were seen within an hour or two after irradiation, continued observation for many hours frequently showed no further change, the remaining vessels continuing to function. Even in an occluded vessel, the architecture of the wall and entrapped cells was maintained with little or no change at 8 hr, and the mass of cells was still visible several hours later. Normal blood flow continued in the surrounding area. Some animals developed arrhythmia and, although circulation became poor, most vessel walls remained intact for many hours.

It was repeatedly noted that, in embryos without a beating heart at 24 hr, the normal structure of small vessels was maintained. This was in sharp contrast to untreated irradiated embryos, in which the lysis and disappearance of vessels occurred rapidly after circulation ceased, frequently within a few minutes.

CONCLUSIONS

The effect of a conditioning radiation exposure in preventing breakdown of the microvasculature following subsequent lethal irradiation resembled the protective effect of soybean trypsin inhibitor. After either type of treatment, radiation frequently resulted in little degeneration and disappearance of small vessels, and normal circulation continued in most vessels for 24 hr and more. Early mortality was reduced by about 50%.

Some years ago we demonstrated a radiation-induced radioresistance in the 3-day chick after exposure to a sub- or minimal-lethal amount of ionizing radiation.⁽⁴⁾ This resistance persisted for several hours and was largest in connection with the mor-

talities expressed within one to two days after irradiation. The nature of the radiation-induced radioresistance has remained obscure. It was previously suggested that the initial radiation exposure resulted in liberation of a substance, the absence of which induced a temporary resistance. Its reformation was then considered to be responsible for the return of the organism toward its original sensitivity.⁽⁴⁾ Results of later experiments, some of which are reported here, indicated that the action of lytic enzymes are important. The specific action of soybean trypsin inhibitor against early radiation injury is assumed to be dependent on its inhibitory action against proteolytic enzymes. In the explanted chick embryo, as well as in the older embryo and the young chick, pretreatment with SBTI reduced early radiation lethality. In embryos treated with SBTI before irradiation, injury to the microvasculature was reduced; the vessel walls frequently showed little or no change over many hours, even in some individuals that did not survive to 24 hr. In other experiments we have demonstrated a cytochemically detectable increase in acid phosphatase at 90 min after a lethal irradiation, suggesting a release or activation of bound enzymes.⁽⁵⁾ In addition, the increase in plasma acid phosphatase activity that occurred at 2 or 3 hr after a lethal irradiation of the young chick was not seen in chicks treated before irradiation with SBTI.⁽²⁾

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RESISTANCE TO EARLY CIRCULATORY DEATH IN THE IRRADIATED CHICKEN: EFFECT OF SPLIT EXPOSURES

S. Phyllis Stearner

PURPOSE AND METHODS

In observations of early radiation mortality (0 to 2 days) in the chick, the comparison of protracted and fractionated exposures (2 or 4 doses) indicated that qualitatively different processes were operating during and between irradiations.⁽¹⁾ The kinetics of injury accumulation during irradiation (protracted exposures) was consistent with an empirical model that postulated a constant or linear reversal of dose effectiveness. There was no evidence of a departure from a linear relation for exposure periods up to 24 hr, and the time constant was comparable in the 6-day chick embryo,⁽²⁾ the 3-day chick,⁽³⁾ and the 6-month rooster.⁽⁴⁾ With split doses, the kinetics of reduction in radiosensitivity to a subsequent irradiation, during the interval after the initial exposure, indicated a nonlinear process. This radiation-induced process, under appropriate conditions (proper magnitude of first dose and interval), resulted in a radiation tolerance above that of the untreated animal and was termed a "resistive process."⁽⁵⁾ Unlike the *reversal* operating during irradiation, the *resistance* appeared to depend on the magnitude of the initial exposure. More recent studies reported here revealed other kinetic differences in the initial lethal response: (1) Throughout development, from the 3-day chick embryo to the 6-month rooster, there was little change in radiosensitivity to single-dose exposures, and the time constants for reversal during irradiation were comparable. (2) The magnitude of the induced resistance increased during development of the chicken. The resistance was relatively small in the 3-day embryo, but in the rooster a relatively refractory state was produced so that the early mortality, a rapidly progressing circulatory failure, did not occur even after a challenging (second) exposure of more than 7000 R γ rays.

The effects of the size of the first (conditioning) fraction of a split exposure to Co^{60} γ rays and duration of the interval between exposures on induced radioresistance in the rooster are described in this report. In addition, comparisons are made with characteristics of the induced radioresistance in the young chick and chick embryo after ionizing radiation.

Roosters (white leghorn) were reared in our animal facilities from cockerels received at 2 days after hatching. At 30 days of age, chicks were wing-clipped and transferred from brooders to cages. They were maintained 2 or 3 to a cage until they were 5 to 6

months of age. Roosters were irradiated in perforated aluminum boxes that were arranged radially around the cobalt-60 source and were rotated at a rate of 0.5 rpm during irradiation in order to equalize the distribution of the delivered dose throughout the body. Single-dose exposures were given in 30 min. The first fraction of a split exposure was given in 15 min, the second fraction in 30 to 45 min. Intervals ranged from 60 min to 24 hr. More than 800 roosters were used in the series of experiments reported here (600 for split exposures and 265 single dose controls). Mortality was recorded daily; survivors after 30 days were sacrificed. All decedents and 30-day survivors were necropsied for observations of gross lesions. Chicks (white leghorn cockerels) were received at 2 days after hatching. At 3 days, they were exposed under standard conditions⁽⁴⁾ to cobalt-60 γ rays. Single-dose exposures were delivered in 10 to 12 min, each fraction of the split exposures was delivered in 12 to 15 min; intervals ranged from 60 min to 24 hr. Mortality was recorded daily for 30 days. Approximately 5000 chicks were included in this analysis.

Fertile eggs (white leghorn) were incubated at 39°C. Embryos, after 3, 6, 12, or 14 days incubation, were irradiated *in ovo* in cardboard boxes. Exposure time for each fraction was less than 15 min. Intervals ranged from 60 min to 24 hr, and eggs were returned to the incubator during this period. Eggs were candled daily until hatching to determine survival. Approximately 3300 embryos were irradiated; of these, the largest series was that exposed at 14 days (2300).

PROGRESS REPORT

Analysis of the influence of dose and duration of interval on early radiation death is based on mortality within 24 hr after the challenging (second) exposure, although survival was observed in all groups for 30 days. Very few deaths occurred on the second day. In all age groups, the largest first dose (600 to 700 R) was at the approximate lower limit of the lethal range (<5%). Radiosensitivity of the rooster, chick, and embryo are shown in Table 16.

The dependence of radiation mortality in the rooster on size of the first fraction and duration of the interval is shown in Figure 30. After a conditioning exposure of 150 R, radiation sensitivity decreased with an increase in interval (Figure 30A), and at 120 min a small resistance (sensitivity less than that

TABLE 16. RADIATION SENSITIVITY OF THE CHICKEN TO EARLY CIRCULATORY DEATH

Age group	Radiation	LD ₅₀ (24 hr) ± S.E., R	Slope ± S.E.	Ref.
Rooster:				
6 mo.	⁶⁰ Co γ rays	1047 ± 17	0.00753 ± 0.00099	(1)
Chick:				
3 days	⁶⁰ Co γ rays	987 ± 12	0.0086 ± 0.0010	3
Embryo:				
14 days	⁶⁰ Co γ rays	792 ± 6	0.01071 ± 0.00084	7
12 days	⁶⁰ Co γ rays	792 ± 25	0.00731 ± 0.00148	7
6 days	⁶⁰ Co γ rays	1011 ± 15	0.00813 ± 0.00177	2
3 days	250 kVp X rays	948 ± 9	0.00698 ± 0.00045	6

(1) This report.

of the unirradiated bird) was present. The resistance was larger after an interval of 240 min, but after a 24-hr interval it had decreased to a level equivalent to that at 120 min. A larger resistance was induced by a first exposure of 300 R (Figure 30B). This was apparent after 120-240 min, but after 24 hr sensitivity was similar to that seen after a first exposure of 150 R. After a conditioning exposure of 600 R (Figure 30C) the resistance was very large at 240 min; a challenging exposure as high as 7400 R resulted in no early mortality. After an interval of 24 hr, however, sensitivity had returned to that observed after the 120-min interval, more than 1000 R greater than in groups that had received either of the smaller conditioning doses. Resistance, as a function of interval for each of the three first-exposure groups, based on estimated LD₅₀ values, is shown in Figure 31. Resistance equal to 0 indicated a sensitivity to the second exposure equal to that in groups not previously irradiated, i.e., effect of the first exposure had been completely nullified.

In the 3-day chick, the effect of a conditioning exposure on subsequent radiation sensitivity was similar to that in the rooster, although the induced resistance was somewhat smaller (Figure 32). Radiosensitivity returned to the preirradiation level (no effect of the first exposure remaining) about 120 min after the initial exposure. With longer intervals radiosensitivity decreased further, and maximum resistance to early radiation mortality (≥ 2000 R) was reached at 240 to 360 min. After 24 hr only a small resistance remained, equal to 200 to 300 R. Unlike the rooster, the resistance at 24 hr showed little relation to the size of the first dose.

In the 14-day chick embryo, a relatively small resistance resulted from an initial exposure of either 300 or 600 R, equal to not more than about 600 R (Figure 33). At 24 hr there was little loss of resistance,

FIG. 30. Dependence of radiosensitivity on size of the first fraction and interval between fractions of a split-dose exposure to ⁶⁰Co γ rays: probit transform of mortality within 24 hr after the second fraction vs. dose by interval group. Least squares constants of the single-exposure group: LD₅₀ = 1047 ± 17 R; slope = 0.00753 ± 0.00099. A, First fraction 150 R. B, First fraction 300 R. C, First fraction 600 R. With 240-min interval no mortality was observed with total exposures up to 8000 R.

so that the total amount remaining was similar to that in the chick. In younger embryos (3 to 12 days' incubation) maximum resistance did not exceed 300 to 400 R.

The effect of age or stage of development on the radiation-induced resistance is shown in Figure 3. Throughout the embryonic period the magnitude of resistance was little changed; the maximum was equal to 500 to 600 R after a conditioning exposure near

FIG. 31.—Rooster: Effect of exposure dose (first fraction) and duration of the interval on resistance to early circulatory death following a second exposure (LD₅₀ values estimated from data in Figure 30). The radiosensitivity of the unirradiated chicken is represented by a resistance equal to zero.

FIG. 32.—Three-day chick: Effect of exposure dose (first fraction) and duration of the interval on resistance to early circulatory death following a second exposure to ⁶⁰Co γ rays.

the lethal level (600 to 700 R) and was largest between 240 and 480 min after the first dose. The 3-day chick developed a much greater radioresistance after a conditioning exposure. The maximum resistance was approximately 2000 R for 50% of the population. Of the remaining 50%, many survived after much larger challenging exposures. In the rooster, the resistance at 240 min after a first exposure of 600 R was very high, and no mortality was observed even after challenging exposures above 7000 R. The resistance was still high (1500 R) at 24 hr, in contrast to the chick and chick embryo in which only about 300 R remained. It is possible that resistance is lost more

FIG. 33. Fourteen-day chick embryo: Effect of exposure dose (first fraction) and duration of the interval on resistance to early circulatory death following a second exposure to ⁶⁰Co γ rays.

FIG. 34. Age effect on radiation induced radioresistance to early circulatory death following a first-dose exposure of 600 to 700 R ⁶⁰Co γ rays (3 day chick embryo exposed to 250 kVp X rays).

slowly in the rooster and that it persists over several days. Tests after longer intervals are required to determine whether or not normal radiosensitivity is ap-

FIG. 35.—Age effect on radiation-induced radioresistance to early circulatory death following a first-dose exposure of 300 R ^{60}Co γ rays.

proached after a longer period. After a smaller first fraction, well below the lethal level (300 R), the induced resistance was approximately the same in the 14-day embryo and 3-day chick, i.e., 200 to 300 R (Figure 35). In the rooster, however, the resistance was large even after this relatively small first dose, i.e., almost 1500 R after 240 min.

CONCLUSIONS

Resistance that can be induced by a conditioning radiation exposure may be characterized as follows: (1) Resistance increases with the magnitude of the first dose. (2) Resistance increases with age after

hatching (14-day embryo < 3-day chick < the rooster), but is unchanged with embryo age (3- to 14-day embryo). (3) In all age groups a resistance can be demonstrated within 180 min after irradiation, and a maximum is reached at 4 to 8 hr. (4) With one exception, an almost identical level of radioresistance (approximately 300 R) is reached at 24 hr in all exposure and age groups; in the rooster, however, the resistance is equal to almost 1500 R. (5) The rate of development of the resistance is nonlinear. (6) There is no relation of the resistance to the single exposure LD_{50} .

The time effects for resistance and reversal processes appear to represent distinct and separate phenomena. There is no quantitative difference in the reversal rate with age (the 3-day embryo and the 6-day rooster are essentially the same), while the resistance is maximum in the rooster and at a similar low level in the 3- to 14-day embryo. Relation of the reversal and resistive processes to the microvascular injury described in living embryos is under investigation.

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EARLY RADIATION INJURY TO THE MICROCIRCULATION: RELATION TO MORTALITY*

S. Phyllis Stearner and Emily J. B. Christian

The early response to lethal doses of ionizing radiation in the chicken and the chick embryo is characterized by rapid structural and functional changes in the microcirculation. Increased vascular permeability and degenerative changes of the vessel walls lead to generalized circulatory stasis and death within a few hours after exposure. These effects were observed in the X-irradiated, explanted 3-day chick embryo and were recorded cinemicrographically for subsequent study. Within 2 or 3 hr after a lethal exposure delivered in a few minutes (1100 R, exposure time 12 min), the vessel walls became swollen, and circulating cells adhered to localized areas of the endothelium. Thrombi formed in such areas, and occlusion of small vessels and degeneration of the vessel walls occurred within 4 to 6 hr after exposure.

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Alterations of the vessel walls were rarely observed after radiation doses below the lethal range. Increasing the exposure time from a few minutes to a few hours decreased the dose effectiveness for this injury mechanism; a larger amount of radiation was required to produce the same effect. Degenerative changes in endothelial cells appeared to be a direct effect, and their incidence was reduced when exposure time was increased.

Early changes in vascular permeability and in the permeability of extravascular structures, such as basement membrane and ground substance, also have been reported after moderate doses of ionizing radiation in mammalian species, including man; usually, these effects are less severe than in the chicken and are not fatal. In newborn rats, however, we have demonstrated an early mortality (<48 hr) after exposures of 600 to 1000 R of X radiation. The presence of edema, hyperemia, and hemorrhages in the central nervous system indicate that vascular injury was a prominent factor in the lethal response.

RADIOSENSITIVITY OF INTESTINAL CRYPT CELLS IN GROUND SQUIRRELS DURING AROUSAL FROM HIBERNATION

Bernard N. Jaroslow, Martha Robbins, and Susan A. Stalling

PURPOSE AND METHODS

Recent work has shown that irradiation of ground squirrels (*Citellus tridecemlineatus*) during hibernation or arousal from hibernation results in increased survival over nonhibernating controls.^(1,2) To develop an understanding of what contributes to increased survival after irradiation at this time, we are studying survival of crypt cells in the ileum and colon of ground squirrels irradiated during and at different times after arousal from hibernation. Hibernating ground squirrels were removed from the cold room, thus initiating arousal. They were irradiated with Co⁶⁰ gamma rays from 0 to 48 hr later, and on the ninth day after irradiation, they were given an intraperitoneal injection of colchicine. Four hours after the colchicine injection, the animals were sacrificed, and pieces of ileum and colon were prepared for histological examination. A sample of the number of surviving crypts was made by counting the number

of crypts in each section that contained epithelial cells blocked in mitosis by the colchicine. This approach was based on the idea of Withers⁽³⁾ for counting colonies of crypt cells in the intestinal wall of irradiated mice.

PROGRESS REPORT

Hibernating ground squirrels were removed from the cold room, and groups of 10 were given 1500, 1600, 1700, and 1800 R at 0, 1, 3, 7, 12, 24, and 48 hr after removal from the cold room. They were maintained at 22°C until they were sacrificed 9 days later. The tissues were fixed, sectioned, and stained, and the number of crypts containing cells in metaphase were counted.

The number of crypts in animals irradiated at 0 and 1 hr after removal from the cold room was higher, at all radiation doses, than their irradiated, nonhibernating controls and it was also higher than in the unirradiated, nonhibernating controls (Figure

FIG. 36 The number of crypts with mitotic figures in three sections of ileum or colon from ground squirrels with colchicine blockade. The standard error is shown. A, The hibernators and their irradiated controls were given 1500 R of ^{60}Co radiation. The untreated controls neither hibernated nor were they irradiated. B, C, and D, Same as A, but radiation dose was 1600, 1700, and 1800 R, respectively.

FIG. 36—continued

36). There are no apparent differences between the hibernators irradiated at the other times and their irradiated controls.

CONCLUSION

These results clearly indicate a difference in the response to irradiation during arousal from hibernation, as compared to fully aroused ground squirrels or the nonhibernating controls. These differences are being investigated.

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KINETICS OF THE IMMUNE RESPONSE INITIATED IN VITRO

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PURPOSE AND METHODS

The study of induction of the immune response has been facilitated by techniques for initiating the immune response *in vitro* that have become generally applicable within the last five years. This breakthrough permits the study of cellular and molecular events associated with the induction and development of the immune response, free from the interaction with concurrent physiological processes *in vivo*. This interaction is likely to become dominant when various antimetabolites or stimulants are used. This becomes obvious in a study of the effects of a drug such as hydroxyurea (see report by the Southwest Cancer Chemotherapy Study Group⁽¹⁾).

Hydroxyurea (HU), as reported by Sinclair,⁽²⁾ killed DNA-synthesizing Chinese hamster cells *in vitro* and blocked those in G_1 from beginning DNA synthesis. These blocked cells could synthesize RNA and protein. If the HU was washed out before the blocked cells were due to divide, they continued their normal growth. If they were exposed to HU beyond their normal division time they died. On the other hand, Pfeiffer and Tolmach⁽³⁾ reported that HeLa cells were not killed by HU added during DNA

synthesis or if they were blocked beyond their normal division time. This deviation from the results with Chinese hamster cells may reflect a difference in HU sensitivity. Effects of HU on the kinetics of antibody-forming cells were investigated in a dissociated spleen cell culture system from normal mice, according to the technique of Mi-hell and Dutton.⁽⁴⁾

The immune response was initiated in a cell suspension (20 million cells/ml) from spleens of unimmunized DBA/1Jax mice stimulated with 100 million sheep red blood cells (SRBC). Each culture contained 1 ml of cell suspension. The number of antibody-forming cells in each culture was determined by the technique of Jerne et al.⁽⁵⁾ The cell suspension is plated on blood agar containing SRBC, and the antibody-forming cells are detected by the appearance of visible clear spots (plaques) in the agar, formed by the hemolytic action of the antibodies that they produce. To remove HU after treatment, the cultures were pooled, washed three times with Hank's salt solution, and divided again into separate cultures. In the progress report that follows, results of a series of experiments designed to investigate the time of onset of DNA synthesis by the precursors of the plaque-forming cells (PFC) and their generation time is measured.

PROGRESS REPORT

Experiment 1

HU was added, at doses from 100 μM to 0.01 μM (Table 17), to the cultures when they were started, and was washed out after 24 hr. Its effect on cell viability, the number of background plaques, and the number of plaques produced in cultures given SRBC after 6 days was small, except perhaps at 100 μM (Group 8). Cells treated with HU for 48 hr were killed.

Experiment 2

HU (0.5 μM) was added to cultures for the following intervals; 0 to 24, 24 to 48, 48 to 96, 96 to 120, and 120 to 144 hr. The dose chosen was the best compromise; it allowed for the production of the maximum number of plaque-forming cells and the minimum toxicity (see Table 17). When HU was washed out, a set of control cultures was given the same wash treatment.

The number of PFC seen on day 6 of culture without SRBC are negligible (Table 18, Group 1).

TABLE 17. EFFECT OF DIFFERENT DOSES OF HYDROXYUREA (HU) DURING THE FIRST 24 HR ON CULTURES STIMULATED WITH SHEEP RED BLOOD CELLS^(a)

Group No.	Mouse spleen cells	Mean No viable cells per culture $\times 10^4 \pm S.E.$	Mean PFC per 10^6 viable cells	
			$\log_{10}$	Anti-log
1	alone	19 ± 1	0.83 ± 0.35	6.8
2	+100 μM HU	18 ± 3	0.0	0.0
3	+10 μM HU	22 ± 4	0.96 ± 0.26	9.1
4	+1.0 μM HU	21 ± 3	0.43 ± 0.27	2.7
5	+0.1 μM HU	28 ± 4	0.49 ± 0.31	3.1
6	+0.01 μM HU	29 ± 4	0.50 ± 0.32	3.2
7	+SRBC	34 ± 6	2.66 ± 0.17	458.0
8	+SRBC + 100 μM HU	8 ± 3	2.42 ± 0.17	263.0
9	+SRBC + 10 μM HU	14 ± 2	3.14 ± 0.06	1380.0
10	+SRBC + 1 μM HU	19 ± 3	3.18 ± 0.11	1510.0
11	+SRBC + 0.1 μM HU	21 ± 5	3.15 ± 0.18	1410.0
12	+SRBC + 0.01 μM HU	19 ± 3	3.02 ± 0.06	1050.0

^(a) All spleen-cell cultures were washed 3 times with Hank's salt solution after 24 hr. The cultures were assayed for plaque-forming cells (PFC) after 6 days.

^(b) Each datum represents the arithmetic average of the $\log_{10}$ values of PFC per 10^6 viable nucleated cells $\pm$ standard error for 5 cultures.

TABLE 18. EFFECT OF PULSES OF HYDROXYUREA ($0.5 \mu M$ HU) ON CULTURES STIMULATED WITH SHEEP RED BLOOD CELLS^(a)

Group No.	Mouse spleen cells	Mean PFC per 10^6 viable cells on day 6 ^(b)					
		Hours when HU was present					
		0 to 24	24 to 48	48 to 72	72 to 96	96 to 120	120 to 144
1	alone	0.0	0.0	0.0	0.0	0.19 ± 0.12 (1.5)	0.49 ± 0.30 (3.1)
2	+SRBC	2.32 ± 0.23 (210)	2.24 ± 0.12 (171)	2.05 ± 0.11 (112)	2.11 ± 0.09 (129)	2.01 ± 0.03 (103)	2.48 ± 0.05 (302)
3	+SRBC + HU	2.66 ± 0.13 (458)	0.92 ± 0.13 (8.3)	0.0	0.0	0.37 ± 0.21 (2.3)	1.30 ± 0.04 (20)

^(a) All cultures were washed as in Table 17 except that the groups were treated with HU at different times during the culture period.

^(b) Each datum represents the arithmetic average of the $\log_{10}$ values of PFC per 10^6 viable nucleated cells $\pm$ standard error for 4 cultures in 0- to 96 hr groups, for 5 in the 96- to 120-hr groups, and for 10 in the 120- to 144-hr groups. Figures in parentheses are the antilog of the mean.

The effect of washing the cultures at different times during the 6 days of culture is small (Group 2). There is no significant difference between the Group 3 cultures given HU for 0 to 24 hr and their washed controls in Group 2. The differences are significant between the HU treated cultures of Group 3 and their respective Group 2 controls for each of the other 24-hr periods.

It is noteworthy that $100 \mu M$ HU, when added to the cell suspensions during the assay procedure, had no effect on the number of plaques counted, i.e., it did not interfere with the synthesis of antibody protein during a one-hour incubation.

Experiment 3

When HU is added to a population of dividing antibody-forming cells in culture, the entire population is killed when the treatment is applied for 24 hr (Table 18), but only cells in the process of DNA synthesis are killed by a short exposure to HU (Sinclair⁽²⁾). This experiment was designed to study the growth kinetics of antibody-forming cells and their precursors.

Three days after the cultures were started and stimulated with SRBC, each group received $38 \mu g$ ($0.5 \mu M$) of HU. After treatments of 2 to 24 hr, the cultures were washed free of HU and maintained as before. After 6 days, the cultures were assayed for PFC. The antilog of the mean $\log_{10}$ PFC for each treatment group divided by antilog for the control group, was calculated and the resulting mean fraction for each HU treatment interval from three experiments were plotted as shown in Figure 37.

HU treatment from 2 to 7 hours shows a 70% decrease in PFC. The number of PFC falls rapidly

FIG. 37.—Survival of the precursors to plaque-forming cells (PFC) after different periods of incubation with $0.5 \mu M$ per culture of hydroxyurea (HU). Each point represents the antilog of the mean $\log_{10}$ PFC per HU treated group divided by that for the control group. This curve is the composite of three experiments.

and approaches zero as the period of incubation with HU increases to 24 hr.

It is not yet clear whether the 2- to 7-hr period should be flat or should have a negative slope.

CONCLUSION

We assume that the fraction of PFC at the time of assay is the same as the fraction of their precursors killed during treatment with HU. We also assume that our results indicate the same effect of HU on our cells as on Chinese hamster cells.⁽²⁾ If these assumptions are correct, our findings indicate little or no DNA synthesis of precursor cells during the first day of culture and active DNA synthesis from then on. At the time of active synthesis, after 3 days, about 70% of the PFC and/or their precursors were synthesizing DNA when HU was added. The shoulder of the curve in Figure 37 indicates, according to Sinclair's interpretation of his data,⁽²⁾ that the be-

ginning of DNA synthesis to division involves slightly more than 7 hr. From the steep slope of the curve, we estimate that the entire cycle is approximately 8-9 hr. This study continues.

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MODIFICATIONS OF ANTIBODY FORMATION

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PURPOSE AND METHODS

Previously, modifications of the primary^(1,3) and secondary^(3,4) Forssman gamma 1 responses in rabbits were reported when sheep red blood cells (SRBC) were injected or reinjected from 4 hr to 2 months after irradiation. The current study, reported in part last year,⁽⁴⁾ deals with modifications of the same two responses when SRBC were injected or reinjected from 1 to 4 hours to 2 months before irradiation. Rabbits were exposed to either a single small (25 to 100 R) or large (500 to 700 R) dose of total-body X rays, and results were compared to unirradiated controls. The foregoing two systematic studies of irradiation have not previously been undertaken for any immune response.⁽⁵⁻¹⁰⁾ Earlier reports on the hemolysin response in rabbits have mainly dealt with the effect of a large dose of X rays at limited intervals on the primary response⁽¹¹⁻¹⁵⁾ and have practically ignored the effect of a small dose on either response.⁽¹³⁾

In unirradiated rabbits after the intravenous injection or reinjection (1.5 to 2.5 months later) of 2×10^8 SRBC per kg rabbit, hemolysin formation⁽¹⁶⁻¹⁹⁾ involves (1) induction during the first few hours, (2) elaboration of the synthetic mechanism during a 2- to 4-day latent period, (3) an acute rise to peak titer, which is attained in a week or two, (4) a rapid decline for several weeks after peak titer, and (5) a

much slower decline for a month or more (see control graphs in Figures 38 and 39). During the last three stages, protein synthesis takes place rapidly at first and more slowly thereafter. To test the effect of X rays on these 5 stages, groups of 9 or more rabbits were exposed to a small dose or a large dose of X rays at 5 appropriate intervals during the primary or secondary response. The individual rabbits in these 20 series were bled 3 or 4 times a week for a month or more, and serums from them were titrated for hemolysin in 50% units by means of a colorimeter.⁽¹⁷⁾

PROGRESS REPORT

Hemolysin titers in individual rabbits in the 20 series were graphed and yielded means $\pm$ standard errors for peak titer and various other parameters of the immune response, including length of the latent period and length and rates of the rise and fall of hemolysin. These means were compared to means $\pm$ standard errors in two unirradiated control series, i.e., for the primary response in 73 rabbits and for the secondary response in 48 rabbits.

The following results were obtained in the rabbits irradiated at critical times during their hemolysin response as compared to unirradiated controls. The small dose of X rays during induction of the primary response stimulated hemolysin formation, as evi-

FIG. 38. Graphs of mean parameters for the primary hemolysin response in 2 series of rabbits irradiated with a small dose of X rays (A and B) and in 2 series of rabbits irradiated with a large dose of X rays (C and D) during induction and during the latent period. Arrows indicate the time of irradiation, and numbers in parentheses indicate the number of irradiated rabbits in each series. For comparative purposes, a control graph of mean parameters for the primary hemolysin response in 73 unirradiated rabbits is repeated in each section.

Note that the mean hemolysin responses in irradiated rabbits are stimulated in series A and B and are at first depressed but eventually enhanced in series C and D.

FIG. 39. Graphs of mean parameters for the secondary hemolysin response in 2 series of rabbits irradiated with a small dose of X rays (E and F) and in 2 series of rabbits irradiated with a large dose of X rays (G and H) during induction and during the latent period. Arrows indicate the time of irradiation, and numbers in parentheses indicate the number of irradiated rabbits in each series. For comparative purposes, a control graph of mean parameters for the secondary hemolysin response in 48 unirradiated rabbits is repeated in each section.

Note that the mean hemolysin responses in irradiated rabbits in series E and F are essentially similar to that in the unirradiated series and are depressed with respect to all parameters in series G and H.

denced by significantly high peak titers attained within normal times and rates (Figure 38, series A). It produced similar results when tested during the latent period (Figure 38, series B), but failed to modify protein synthesis when tested during the rise and fall of hemolysin, i.e., at 4, 9, and 21 days after the initial injection of antigen. Different results, as might be expected, were obtained with 500 to 700 R of X rays. This large dose of X rays, when tested during induction of the primary response at first depressed but eventually enhanced hemolysin formation, as evidenced by a longer latent period, a longer and slower rise to peak titer which culminated in a significantly high peak titer and a slow decline after peak titer (Figure 38, series C). It produced similar spectacular results when tested during the latent period (Figure 38, series D). Subsequently, when this large dose was tested at 4, 14, or 40 days after the injection of SRBC, depression progressively overshadowed enhancement. For example, when rabbits were exposed to a large dose of X rays 40 days after they had received SRBC, hemolysin declined more sharply and more erratically after than before irradiation and increased in titer only slightly and occasionally.

Irradiation effects on peak titer during the secondary response fell far short of those occurring during the primary response. A small dose during induction or during the latent period of the secondary response did not produce any statistically significant stimulating effect (Figure 39, series E and F), whereas a large dose during the same early stages of the secondary response was predominantly depressing as evidenced by delayed and subnormal peak titers (Figure 39, series G and H).

CONCLUSIONS

These data on immunization *before* irradiation, in agreement with our recent study on immunization *after* irradiation, suggest that the immunologically competent initial cells may be more easily stimulated and may recover from injury more easily than the activated memory cells which are present at the time of reinjecting SRBC because of prior initial antigenic stimulation.

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DEVELOPMENTAL DELAY AND LETHALITY STUDIES OF X-IRRADIATED TRIBOLIUM CASTANEUM EGGS

Tracy Chui-Hsu Yang* and George A. Sacher

PURPOSE AND METHODS

A mathematical theory of recovery in turning-over cell populations after irradiation was proposed by Sacher and Trucco.⁽¹⁾ This theory is based on the assumptions that the recovery rate is proportional to the number of proliferative cells that survive irradiation, and also proportional to the amount of recovery still to be completed, i.e., to the difference between the existing population size and the set point. This theory generates an explicit mathematical model of recovery, which has the form of a logistic equation. One prediction of the model is that the time needed for the population to recover to a specified size is proportional to the radiation dose. The predictions of the model are in accord with data from split-dose recovery studies. The cell-recovery model studies by Sacher and Trucco contained no term for the delay of mitosis in the surviving proliferative cell population. Sacher subsequently developed a model for division delay,⁽²⁾ which yields a second-order kinetic model for the inactivation and regeneration of a substance essential for recovery in agreement with observations on synchronized mammalian cells. This model predicts that division delay is proportional to dose. Hence, both of the major terms in recovery, that for intracellular recovery from division block and that for the restoration of cell numbers, give rise to a proportionality of delay to dose.

The insect egg is a closed system, so the hatching delay will reflect the growth delay of the embryo. Information on the hatching delay of irradiated insect eggs will, therefore, be valuable for testing the model as well as for understanding the radiation effects on development. Radiosensitivity of *Tribolium* eggs at different ages was also studied.

Adult beetles, less than one month from eclosion were put in a fresh flour-yeast medium for 1 hr at 30°C and then were sifted from the medium and eggs. The time when the adults were removed was counted as zero hr for the age of eggs. After separation from medium by a finer sieve, the eggs were kept in tissue culture dishes at 24°C until they hatched. The number of larvae that hatched was checked every 8 hr from the sixth to tenth day. Eggs which failed to hatch before the tenth day were considered to be dead. Irradiation was with a General Electric

deep therapy X-ray unit at a dose rate of 105 R/min. Exposures were at 250 kV, 15 mA, and half-value layer of 1.5 mm of copper.

PROGRAM REPORT

The hatching delays for *T. castaneum* eggs X irradiated at different ages are presented in Figure 40. The relative hatching time was calculated by the equation

$$\frac{\left(\frac{\sum n_i t_i}{\sum n_i}\right)_{\text{irrad}}}{\left(\frac{\sum n_i t_i}{\sum n_i}\right)_{\text{control}}}, \quad (1)$$

where n_i is the number of eggs hatched at time t_i .

Results with 6-hr-old eggs indicate that the development delay is proportional to the dose. According to Sacher and Trucco, the growth of an undisturbed population proceeds at a constant rate, ρ , in the absence of external factors limiting growth. The accumulated number of divisions at time, t , after dose D , Q_{dt} is given by Sacher⁽²⁾ as

$$Q_{dt} = \rho \int_0^t \frac{1}{1 + (e^{kD} - 1)e^{-kt}} dt \quad (2)$$

$$Q_{dt} = \rho \left\{ t - \frac{KD}{k} + \frac{1}{k} \ln [1 + (e^{kD} - 1)e^{-kt}] \right\}, \quad (3)$$

where k is a rate constant, and K an inactivation constant.

The deficit of number of division, Δ_{dt} , is

$$\Delta_{dt} = Q_{dt} - Q_{dt} = \frac{\rho}{k} \{ Kd - \ln [1 + (e^{kD} - 1)e^{-kt}] \}, \quad (4)$$

FIG. 40 - Hatching delay of *Tribolium castaneum* eggs X irradiated at different ages.

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and the delay in reaching an assigned number of divisions, L_{Dt} , is calculated by

$$L_{Dt} = \frac{1}{\rho} \Delta_{Dt}. \quad (5)$$

The interval between irradiation of *Tribolium* eggs and the hatching of larvae is sufficiently long so that the asymptotic form of Equation 4 can be used:

$$\begin{aligned} L_{D\infty} &= \frac{1}{\rho} \Delta_{D\infty} \\ &= \frac{1}{\rho} \left(\frac{\rho K D}{k} \right) \\ &= \frac{KD}{k}. \end{aligned} \quad (6)$$

This equation indicates that the hatching delay is proportional to the dose. The relation of delay in dose is linear at all ages, but a shoulder is observed in the hatching delay of eggs 1 day old and older at irradiation. According to the above equation, there should be no shoulder if cell killing is exponen-

tial. It is possible that the extrapolation number for the killing of embryonic cells, or nuclei, is near unity at early ages and increases when differentiated embryonic cells appear.

The Sacher-Trucco theory is most directly applicable to systems of undifferentiated cells, e.g., tissue culture cells, unicellular organisms, etc. Our histological studies indicate that: (a) the 6-hr *T. castaneum* egg is at cleavage stage, after having had a series of endomitotic nuclear divisions; (b) the 1-day-old egg has passed the blastoderm stage; and (c) many organs have been formed in 3-day-old eggs. The 6-hr egg is, therefore, a single cell with multiple nuclei, and the 1-day-old egg contains many differentiated cells.

The results also show that the slope of the hatching delay function is not the same for eggs of different ages. This difference may be due to the fact that the inactivation constant, K , and the rate constant, k may vary in different types of cells.

Figure 41 depicts the effect of X irradiation on the survival of *T. castaneum* eggs. It was found that the 6-hr-old egg is most sensitive to radiation, that the 1-day- and 2-day-old eggs are less sensitive, and the 3-day-old eggs are least sensitive. The slope at LD_{50} is about the same for eggs of all ages, indicating that the number of targets that must be destroyed to kill the egg increases with the age of the egg.

CONCLUSIONS

Hatching delay for *T. castaneum* eggs irradiated at age 6 hr is directly proportional to the radiation dose. There is a shoulder for eggs irradiated at later stages, and the slope of the curve also changes with the age of eggs. X irradiation is more effective in delaying or preventing hatching in the early, cleavage, stages than in the later, differentiation and growth, stages.

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FIG. 41.—Survival curves of *Tribolium castaneum* eggs X irradiated at different ages.

EFFECTS OF X IRRADIATION ON SOME PHYSICAL PROPERTIES OF A DEVELOPING TRIBOLIUM

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PURPOSE AND METHODS

These studies explored the mechanism of the radiation effects on physical properties of the developing flour beetle (*Tribolium castaneum*). Changes of body weight, elytrum† weight, and mechanical strength of elytra in relation to radiation, as well as the kinetics of recovery, were examined.

Two-week old larvae, raised in 4% flour-yeast medium at 30°C and a relative humidity of 60 to 70%, were irradiated with a General Electric deep therapy X-ray unit at a dose rate of 450 R/min. Physical conditions of irradiation were 250 kV, 15 mA, and half-value layer of 1.5 mm of copper. One week after the beetles emerged as adults, they were weighed alive on a microbalance to obtain the wet body weight. Elytra dissected from the beetles were weighed on the same balance. In order to determine the mechanical strength of an elytrum, a materials-testing instrument, the Instron‡ was used. An elytrum was placed, with its inside surface facing up, on an L-shaped aluminum holder whose upper end was connected to a tension-measuring load cell on the upper part of the instrument. A thin (0.1336-mm diameter) wire was placed across the midpoint of the long axis of the elytrum. With one end at each side of the elytrum, both ends of the wire were passed through a slit (0.5 mm wide and 0.5 cm long) located under and at a 90° angle to the long axis of the outer wing. The wire then was connected to the lower jaw of the instrument which was pulled away from the elytrum, causing the wire to stretch, and eventually, break the elytrum. The force applied to the elytrum before it broke was automatically registered on a recorder.

PROGRESS REPORT

Figure 42 shows the effect of X irradiation on the body weight of adult *T. castaneum*. The curve had a shoulder at 1 kR; body weight decreases with an increase in dose up to 3 kR. In this study, adults that emerged had a body weight below 1.4 mg when the X-ray dose was over 3 kR. When the 3-kR dose was split into two equal fractions and given to the 2-week-old larva at different intervals, there was a

rapid recovery of body weight of the adults. The kinetics of recovery is similar to that in survival studies,⁽¹⁾ i.e., recovery of an organism takes place rapidly during the first few hours, drops to a minimum at 6 hr, and then rises progressively.

Elytrum weight had the same type of response to X irradiation as body weight (see in Figure 43). A minimum of recovery at 6 hr was again observed. There was difference in radiosensitivity between the left and right elytrum, and a dose of 3 kR resulted in about a 28% decrease in weight. Both the body-weight and the elytrum-weight studies indicate that radiation has an inhibitory effect on the development of adult organs and tissues.

FIG. 42.—Radiosensitivity and recovery of body weight of X irradiated *Tribolium castaneum* adults. Standard error is shown for each point.

FIG. 43.—Effect of X irradiation on the elytrum weight of adults.

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† Modified horny anterior wings.

‡ Instron Engineering Corporation.

Figure 44 depicts the change of mechanical strength of elytrum of an adult *X* irradiated when it was a 14-day-old larva. A threshold was found at 1 kR, and the mechanical strength diminishes as the dose is

FIG. 44.—Effect of X irradiation on the mechanical strength of the elytrum, expressed as the total force, in grams, required for breakage. Left, dose effect relationship; right, split-dose recovery function.

FIG. 45.—Effect of X irradiation on the mechanical strength of the elytrum, expressed as g/mm of elytrum width. Left, dose-effect relationship; right, split-dose recovery function.

increased. The kinetics of recovery of mechanical strength of elytrum, the most interesting finding in this study, was found to be an exponential type with no complicated pattern, i.e., there was no minimum at 6 hr. When the mechanical strength of the elytrum is expressed as g/mm, as shown in Figure 45, no minimum was found at 6 hr in the time course of recovery, although the left elytrum did exhibit a slight drop of recovery at 4 hr. The difference between the kinetics of recovery of survival, body weight, and elytrum weight and that of mechanical strength of elytrum may be due to the fact that survival, body weight, and elytrum weight are directly related to the number of cells in the organism, but the mechanical strength of elytrum is related more closely to the amount of cuticle (chitin) secreted by cells in the elytrum. The recovery minimum that appears at 6 hr is probably due to a phase of radiosensitivity in the cell mitotic cycles of the partly synchronized cell population that emerges from mitotic block.⁽²⁾ The exponential course of recovery of mechanical strength of elytrum after X irradiation is not inconsistent with the hypothesis that the loss of strength is due to cytoplasmic injury. The present results indicate that the radiosensitivity of cytoplasm may not change measurably during cell mitotic cycle and that the recovery of cytoplasm follows first order kinetics, i.e., $dS/dt = -kS$, where S is the amount of injury at time t .

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THE LETHAL EFFECTS OF MONOENERGETIC NEUTRONS AND ⁶⁰Co GAMMA RAYS ON TRIBOLIUM CASTANEUM

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PURPOSE AND METHODS

As part of an ongoing program for determination of neutron toxicity and RBE, the radiosensitivity of *Tribolium castaneum* larvae was determined at several neutron energies. Current microdosimetry theory predicts that maximum neutron RBE will be found near 0.4 MeV.⁽¹⁾ This prediction is difficult to verify for organisms much larger than a few grams

because of the neutron energy degradation which occurs in tissue and the difficulty of obtaining uniform dose and energy distributions over the entire body. The larvae, however, are so small that these problems are eliminated; therefore, they seemed a good choice for examination of the variation of RBE with neutron energy.

Larvae were exposed in thin-walled polyethylene vials containing 100 larvae each in about 1 gram of support media.⁽²⁾ Vials were mounted, 12 at a time,

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on a thin aluminum plate which was rotated at 1 rpm during exposure in the monoenergetic neutron facility previously described.⁽³⁾ The vials were positioned symmetrically in a plane 51 mm from the source, at a perpendicular distance of 51 mm from the axis of the proton beam. Thus, vials were located on a circle making a 45° angle with the beam at a radial distance of 72 mm from the source. Target thicknesses ranged from 95 to 125 keV, and dose rates were about 3 Rads min⁻¹. Conditions of growth, maintenance, and lethality scoring were as previously described.⁽²⁾

Larvae were also exposed to ⁶⁰Co γ rays at different dose rates, and lethality were scored as previously described.⁽²⁾

PROGRESS REPORT

Complete dose-effect curves were obtained at 663 keV and 439 keV, with two sets at the lower energy. These are shown in Figure 46 and may be compared with the ⁶⁰Co γ curves of Figure 47. Preliminary results have also been obtained for 104, 170 and 1319 keV, and these are given in Table 19 along with computed RBE values.

CONCLUSION

Despite the fragmentary data at 104 and 1360 keV, the results obtained, combined with the Janus

FIG. 46.—Dose-response curves of neutron-irradiated *Tribolium* larvae. Each point represents the mean survival of 100 to 200 larvae. Two experiments were performed at 439 keV.

FIG. 47.—Dose-response curves of *Tribolium* larvae irradiated with ⁶⁰Co gamma rays at various dose rates. Each point represents the mean survival of 200 larvae.

TABLE 19. LETHALITY AND RBE VALUES FOR *T. Castaneum*

Neutron energy, KeV	30-day lethality			RBE ^(a)
	ϵ , LD	Dose, Rads	Dose rate, Rads/min	
Janus ⁽³⁾	50	938	50	4.1
104	5	900	0.3	5.0
170	50	810	3	7.6
439	50	620	3	10.0
663	50	1150	3	5.1
1319	15	1050	3	4.5

^(a) The ratio of neutron to γ doses for the same ϵ , LD and dose rate. ⁶⁰Co γ LD₅₀ was 3810 Rads at 50 Rads/min and 6200 Rads at 3 Rads/min. ⁶⁰Co Rads are actually roentgen exposure values; neutron Rads are Kerma to Standard Man.⁽²⁾

neutron results previously reported, show clearly that maximum RBE occurs around 400 keV as predicted. With the forthcoming availability of a Dynamitron monoenergetic facility of greatly increased dose rate, we hope to examine the radiosensitivity of these larvae from 0.03 to 19.2 MeV at various dose rates and to examine carefully response at the N and O neutron resonances to verify or deny our hypothesis⁽²⁾ of specific chemical effects there.

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STUDIES ON THE DEVELOPMENT OF MID-GUT OF THE TRIBOLIUM CASTANEUM ADULT

Tracy Chui-Hsu Yang and Everett Staffeldt

PURPOSE AND METHODS

Since *Tribolium* was used in nutritional studies by Chapman (1924), considerable attention has been given to the flour beetles by ecologists, geneticists, and radiobiologists. While ecological, genetic, and radiobiological information on *Tribolium* has increased rapidly in the past 20 years, developmental information has been limited to the external body structures.

The physiological cause of death of irradiated *Tribolium* has long puzzled radiobiologists.^(1, 2) Because this organism is small and has a hard cuticle, physiological and histological studies of *Tribolium* are very difficult.

Despite these difficulties, we studied the development and structure of *Tribolium castaneum* mid-gut by histological methods and examined the effects of radiation on its development.

The general method of handling *Tribolium castaneum* has been reported before.⁽³⁾ When they reached late larval or pupal stage, they were fixed for 7 days in a mixture of a saturated solution of picric acid

in 90% ethyl alcohol, 75 parts; formalin, 25 parts; concentrated nitric acid, 8 parts. After fixation, the beetles were washed in warm 70% ethyl alcohol for several minutes. Dehydration was accomplished through a series of graded ethyl alcohols. Clearing was not necessary. They were then embedded in celloidin and paraffin with the conventional ether-alcohol-celloidin mixture for 7 days. The blocks were hardened overnight in chloroform. The block was trimmed into a small square with the beetles in the center. To insure proper infiltration of paraffin into celloidin, the blocks were promptly transferred to a suspension of 1 part paraffin, and 5 parts chloroform, in which they were kept overnight at room temperature (24°C).⁽⁴⁾ Sections were cut at 5 and 10 μ and were stained with Malloy's triple stain and Mayer's hematoxylin and eosin.

In order to investigate the effect of radiation on the development of the *T. castaneum* mid-gut, 2-week-old larvae were irradiated with a General Electric, deep therapy X-ray unit, Maximar. Physical conditions of irradiation were 250 kV, 15 mA, and

FIG. 48.—A cross section through the mid-gut of 14-day-old larva. (M) malpighian tube, (L.I.) mid-gut of larval intestine. Fixation, nitric acid-picric acid; staining, Mayer's hematoxylin and eosin.

FIG. 49.—A longitudinal section of pupa. (P.I.) pupal intestine, (L.I.) undigested larval intestine. Fixation, nitric acid-picric acid; staining, Mayer's hematoxylin and eosin.

half-value layer of 1.5 mm of copper. About one week after emerging as adults, the beetles were sacrificed for histological examination.

PROGRESS REPORT

A cross section of mid-gut of a 14-day-old larva is shown in Figure 48. Epithelial cells are packed together and appear to fill the intestinal lumen. This cellular arrangement may benefit the larva by greatly increasing the surface area of its intestine. When *T. castaneum* reached its pupal stage, there was a change of structure of mid-gut. While the

FIG. 50 A longitudinal section of pupal mid intestine at a higher magnification. (L.I.) a piece of undigested larval intestine, (Nd) midgut of pupal mid gut, (P.I.) pupal intestine, (I.N.) nucleus of intestinal epithelial cell. Fixation, nitric acid-picric acid, staining: Mayer's hematoxylin and eosin.

FIG. 51 A cross section of adult (C) cecum of mid gut, (M) malpighian tube. Fixation, picric acid-alcohol-formalin-nitric acid, staining, Mallory's triple stain.

FIG. 52 A cross section of mid gut of flour beetle X irradiated at 14 day-old larval stage. Dose, 4 kR, dose rate, 450 R/mm. (C) cecum of mid intestine. Fixation, picric acid-alcohol-formalin-nitric acid, staining, Mallory's triple stain.

intestine of the pupa was forming, the larval mid-gut epithelial cells were extruded into the lumen, where they degenerated, as shown in Figure 49. The newly formed pupal intestine, containing a piece of undigested larval mid-gut, is shown in Figure 50 at higher magnification. The nuclei in the pupal intestinal epithelial cells appeared to be *small* in size, and the cells form a continuous layer. Some mid-gut cells, however, aggregated to form mounds. In the adult these cells develop into caeca. A typical cross section of adult mid-gut is shown in Figure 51. Epithelial cells in the mid-gut of the adult appeared to be cuboidal and have *large* nuclei. The cecum of *T. castaneum* appeared to be very similar in structure to that found in *Thanatophorus japonicus*, a Coleopteron, by Shimoda.⁽⁵⁾ Mitosis in the cecum was demonstrated by a squash preparation.

When the structure of the mid-gut of a week-old *T. castaneum*, X irradiated with 4 kR at the 14-day-old larval stage was examined, the cecum was seen to be almost denuded of epithelial cells, as shown in Figure 52. Although it appears that radiation killed these epithelial cells, it may not be completely responsible.

CONCLUSIONS

The structure of the mid-gut of *Tribolium castaneum* was found to be different at different stages of its development. The cecum, not present in the larval stage, was developed in adult mid-gut, and some mitotic activity was observed. X irradiation given at the larval stage caused some damage to the development of the adult mid-gut.

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STATEMENT OF PROGRAM OBJECTIVES

*Robert J. Flynn, Thomas E. Fritz, Calvin M. Poole,
Ronald W. Camden, and Patricia C. Brennan*

Proper care of laboratory animals is both a humane and a scientific requirement. Unfortunately, objective experimentally-obtained information is often lacking on what constitutes proper care. The Division's research program in laboratory animal medicine is aimed at meeting this need. This program develops improved methods for the production and management of the animals used in the Division's research programs. Research directed toward solving production problems is illustrated by reports of the age of laboratory rodents at time of first fertile mating; the development of an inbred acatalasemic mouse; *Rattus rattus* as a research animal; genetic, nutritional and environmental effects on Syrian hamster production; a coat color mutation in the Syrian hamster; and monogamous mating of Chinese hamsters. Additional research concerns the refinement of

controlled management. This phase is illustrated by the report on frequency of cage cleaning and the survival of mice. A special effort is devoted to studying management of laboratory animal disease, particularly those diseases that represent hazards to the Division's research programs involving animals. This phase is illustrated by reports on hepatic and renal lesions in a hamster breeding colony and throat flora of a closed colony of beagle dogs.

This program also provides cooperative, specialized professional assistance (pathology, microbiology, roentgenography, surgery) to the Division's research program. This support is illustrated in reports found elsewhere in this publication (see the sections on Mechanisms of Carcinogenesis, Radiation Protection, and, particularly, the Toxicity and Metabolism of Radionuclides).

THE SUPPLY AND MAINTENANCE OF DEFINED ANIMALS FOR THE DIVISION'S RESEARCH PROGRAM: STATUS OF THE COLONY

*Calvin M. Poole, Ronald W. Camden, Thomas E. Fritz,
Patricia C. Brennan, and Robert J. Flynn*

PURPOSE AND METHODS

High quality animals are necessary for effective biomedical research. The specific animal requirements for different research projects is extremely diverse and variable, and the general trend is toward the use of animals that are more highly controlled and defined. It is the purpose of the animal facilities group to provide animals that are consistent with these requirements through production or acquisition and to furnish compatible maintenance. The methods used to provide and maintain such animals have been reported.⁽¹⁻⁹⁾

maintained in the Division's animal facilities during the past fiscal year are presented in Table 20.

The studies in which these animals have been used during this period may be determined by reference to other parts of this report. Animals produced within the Division's facilities are designated with the suffix "Anl" (for Argonne National Laboratory).

There have been no serious disease outbreaks in the past year. Isolated episodes of rodent respiratory diseases and definition of the causes of canine neonatal death appear to be the most important disease problems within the animal facilities at this time.

PROGRESS REPORT

The June 30, 1969, animal inventory and the number and kinds of animals produced or acquired and

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TABLE 20. ANIMAL INVENTORY

Common name	Scientific name	Inventory as of June 30, 1969	Total acquired July 1, 1968- June 30, 1969	Total produced at Argonne July 1, 1968-June 30, 1969
Mouse	<i>Mus musculus</i>			
Germfree		0	120	0
SPF		20,821	0	71,261
Conventional		6,361	3,741	13,366
Whitefooted mouse	<i>Peromyscus leucopus</i>	228	0	180
Brush mouse	<i>Peromyscus boyleyi</i>	32	0	6
California mouse	<i>Peromyscus californicus</i>	114	0	30
Canyon mouse	<i>Peromyscus crinitus</i>	29	0	0
Cactus mouse	<i>Peromyscus eremicus</i>	18	0	0
Florida mouse	<i>Peromyscus floridans</i>	41	0	45
Cotton mouse	<i>Peromyscus gossypinus</i>	111	0	60
Golden mouse	<i>Peromyscus nuttalli</i>	1	0	0
Desert pocket mouse	<i>Perognathus penicillatus</i>	4	0	0
Harvest mouse	<i>Reithrodontomys humilus</i>	47	0	20
Vole	<i>Microtus</i> sp.	11	11	10
Tunisian jird	<i>Meriones libycus</i>	1	0	0
Rat	<i>Rattus norvegicus</i>	2,896	300	8,191
Black rat	<i>Rattus rattus</i>	69	9	75
Multimammate mouse	<i>Praomys</i> (Mastomys) <i>natalensis</i>	948	0	1,892
Cotton rat	<i>Sigmodon hispidus</i>	63	0	120
Pack rat	<i>Neotoma albigula</i>	3	0	0
Rice rat	<i>Oryzomys palustris</i>	5	0	60
Tree shrew	<i>Tupaia terruginea</i>	1	0	0
Syrian hamster	<i>Mesocricetus auratus</i>	1,617	348	4,096
Chinese hamster	<i>Cricetulus barabensis</i>	384	0	796
Turkish hamster		6	0	1
Chinchilla	<i>Chinchilla laniger</i>	10	0	0
Chipmunk	<i>Tamias striatus</i>	8	0	0
Ground squirrel	<i>Citellus tridecemlineatus</i>	60	429	0
Tropical red squirrel	<i>Sciurus granatensis</i>	2	0	0
Guinea pig	<i>Cavia porcellus</i>	0	86	0
Rabbit	<i>Oryctolagus cuniculus</i>	60	174	0
Dog (beagle)	<i>Canis familiaris</i>	689	0	142
Monkey	<i>Macaca mulatta</i>	1	0	0
Chicken	<i>Gallus domesticus</i>	425	2,000	0
Frog	<i>Rana pipiens</i>	0	1,200	0
Snail	<i>Helix aspersa</i>	45	0	0
Grasshopper	<i>Melanoptus differentialis</i>	1,000	0	4,000

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AGE OF LABORATORY RODENTS AT TIME OF FIRST FERTILE MATING

Ronald W. Camden, Robert J. Flynn, and Calvin M. Poole

PURPOSE AND METHODS

These studies were initiated to determine the earliest ages at which fertile mating may occur in our strains of rodents.^(1,2) We have reported that female CF#1, Anl[Anl 66] mice are capable of fertile mating as early as 26 days and the males as early as 40 days of age. Fertile matings occurred as early as 23 days of age in CP Anl[Anl 66] females and 44 days in the males; 25 days of age in CN-1 Anl[Anl 66] females and 43 days in the males; and 34 days of age in C57BL 6Anl[Anl 66] females and 41 days in the males. In addition, a fertile mating occurred at 19 days of age in a CP Anl female and 20 days of age in a CN-1 Anl female.

Reports in the literature of age at sexual maturity in mice are extremely variable. The earliest age of vaginal opening reported in the female is 24 days, in a C57BL₆ J.⁽³⁾ Vaginal cornification does not occur for 24 to 120 hr after vaginal opening, and willingness to mate is sometime later. No reports deal specifically with the age at first fertile mating in either sex, and little is reported about sexual maturity in the male.

The age, in the literature, of female rats at sexual maturity is quite variable.⁽⁴⁾ The youngest mean age reported for the Sprague-Dawley female rat at time of vaginal opening is 39.6 days. The youngest mean age reported for a female rat, of any strain, is 38 days. No reports concern the male, except that sexual maturity occurs later than in the female.⁽⁴⁾

During the period covered by this report, we studied C3Hf Anl[Anl 66] and DBA 1Anl[Anl 66] mice and SD Anl[Anl 66] rats. Matings of 20- or 21-day-old males or females with adults of the opposite sex were made, and the birth date of the first litter was recorded. The adults used were near 60 days of age. In the case of the rats, matings of young males (near 30 days of age) with females of a comparable age were also made.

The age of an individual animal at its first fertile mating was obtained by subtracting the mean length of the gestation period, determined in separate studies to be 19 days in our mice⁽⁵⁾ and to be 22 days in our rats,⁽⁶⁾ from that individual's age when its first litter was born.

PROGRESS REPORT

The ages at first fertile mating for C3Hf Anl[Anl 66] and DBA 1Anl[Anl 66] mice are given in Table

21. The earliest ages for the females are 23 and 25 days for the respective strains, and the average ages are 28.1 and 32.9 days, respectively. The earliest ages for the males are 39 and 41 days, respectively, and the average ages are 48.0 and 47.9 days.

Table 21 also gives the ages at first fertile mating for female and male SD Anl[Anl 66] rats. The earliest age for the female is 25 days, and the average age is 30.3 days. The earliest age for the male is 49 days. The average age at first fertile mating for males paired with 60-day-old females is 73.5 days, and for males paired with 30-day-old females it is 63.4 days. The number of infertile matings is higher when using 60-day-old females (13 of 64) than when using 30-day-old females (2 of 61).

CONCLUSIONS

The earliest age of the first fertile mating of female mice reported here is earlier than would be possible from the ages of vaginal opening reported in the literature. There is a considerable difference between the average ages of females from the two different strains. Although this may be a genetic difference, data for males of the two strains are similar.

The earliest age and the average age at first fertile matings for female SD Anl[Anl 66] rats are much lower than any reported in the literature. Male rats are more than twice as old as females at time of their first fertile mating. Mating young males with females of a comparable age (Male B in Table 21) seems to provide a better test system than mating with adult females (Male A).

TABLE 21 AGES AT FIRST FERTILE MATING FOR C3Hf Anl[Anl 66] AND DBA 1Anl[Anl 66] MICE, AND SD Anl[Anl 66] RATS

Strain	Sex	Number observed	Number fertile	Age range, days	Average age, days
C3Hf Anl[Anl 66]	Female	29	28	23-39	28.1
DBA 1Anl[Anl 66]	Female	22	22	25-57	32.9
C3Hf Anl[Anl 66]	Male	41	35	39-82	48.0
DBA 1Anl[Anl 66]	Male	23	21	41-57	47.9
SD Anl[Anl 66]	Female	60	60	25-45	30.3
SD Anl[Anl 66]	Male A ^a	64	51	49-148	73.5
SD Anl[Anl 66]	Male B ^b	61	59	52-84	63.4

^a 21 day-old males mated with 60 day-old females.

^b 30 day-old males mated with 30 day-old females.

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THE DEVELOPMENT OF AN INBRED ACATALASEMIC MOUSE

Ronald W. Camden, Robert N. Feinstein, and Robert J. Flynn

PURPOSE AND METHODS

The acatalasemic mutant of the mouse (Cs^b) is being introduced into the C3Hf Anl mouse in order to provide an acatalasemic animal of uniform coat color, size, and growth rate with good reproductive performance.^(1,2) This is being done by initial crosses between acatalasemic mutants and C3Hf Anl mice and subsequent backcrosses between mutant carriers and the C3Hf Anl stock. Only mutant carriers with low catalase levels and from large litters (8 or 9 weaned) are used for further breeding. Eight generations of backcrosses and subsequent sibling matings between mutant carriers are required to produce an inbred acatalasemic mouse (C3Hf Anl- Cs^b) that is genetically comparable to the normal catalasemic C3Hf Anl mouse.

PROGRESS REPORT

The eight backcross generations and one sibling mated generation have been completed and we have

24 breeding pairs in F_2 matings. The resultant mice are uniform in body size and coat color and reproduce well.

CONCLUSION

We have introduced the Cs^b locus into C3Hf Anl mice and have produced mice of uniform coat color and body size that are acatalasemic and reproduce well.

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RATTUS RATTUS AS A RESEARCH ANIMAL

Thomas E. Fritz, David V. Tolle, Patricia C. Brennan, Richard C. Simkins, and Robert J. Flynn

PURPOSE AND METHODS

Recent criticism of the albino rat (*Rattus norvegicus*) as a laboratory animal⁽¹⁾ and confusing data on the relationships of the laboratory rats with wild strains of *R. norvegicus* and *R. rattus*⁽²⁾ prompted us to establish a colony of the "roof" or "black" rat (*R.*

rattus) and to compare it with the albino laboratory rat.

PROGRESS REPORT

Initial attempts to acquire live specimens through commercial animal suppliers and laboratory animal raisers were frustrating. No supplier or raiser of

rodents listed in a comprehensive source directory⁽³⁾ supplies either specimens or information about where they could be obtained.

Through extensive personal communications and inquiries, we finally located a source of *R. rattus* at the National Communicable Disease Center, Technical Development Laboratories, Savannah, Georgia. This laboratory maintains an uncaged, roof-rat colony in a shed. The original stock for this colony was collected from sylvatic sources.

A first group of three trios (6 females and 3 males) of roof rats survived no longer than 10 days in our laboratory. They were housed like our albino rats, individually, in plastic cages fitted with stainless steel tops. Although they were given a variety of food (including a standard laboratory rodent feed, fresh fruits and vegetables, canned dog food and dry powdered milk and water) none ate or drank, and all literally starved to death.

A second group of 9 animals was caged as trios in large wire cages, approximately $4 \times 3 \times 21\frac{1}{2}$ feet. They had wooden nesting boxes, wood shavings for bedding and were fed a standard laboratory rodent feed and canned dog food, plus water. These rats survived and were kept in this environment for approximately 9 months, with 6 litters born during this time. After 9 months, all but 2 pairs were put into plastic cages as monogamous pairs. During the past 4 months, 15 additional litters have been born for a total of 21 litters in 13 months. The 21 litters contained 131 animals, 83 of which have been weaned. Twenty-eight animals are still suckling and the re-

mainder have died. Cannibalism has been the major cause of death.

This breeding colony now has 24 breeding pairs in plastic cages, and two pairs are in wire cages. At this time, 3 litters of weanlings have not been grouped as breeding pairs.

Deaths among the adult and weanling animals have resulted from complications of cutaneous lacerations due to fighting or respiratory infections. Handling problems that have resulted in fatal trauma are due to the particularly nervous attitude of the rats and their violent attempts to escape from any type of restraint. Respiratory disease has been associated with infections of both *Mycoplasma pulmonis* as well as *Pasteurella pneumotropica*.

CONCLUSIONS

A production colony of *R. rattus* is feasible. We hope that *R. rattus*, particularly its first- and second-generation offspring, will adapt to its new environment well enough so that we can produce a number that will allow studies of its biological characteristics and permit comparisons with the standard albino laboratory rat.

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GENETIC, NUTRITIONAL, AND ENVIRONMENTAL EFFECTS ON SYRIAN HAMSTER PRODUCTION

Ronald W. Camden, John E. Van Boskirk, Allen B. Reiskin,
Robert J. Flynn, and Calvin M. Pool

PURPOSE AND METHODS

To determine the reason for lower production rates in the Division's Syrian hamster colonies compared with the rates reported by commercial breeders, studies of the effects of genetic, nutritional, and environmental variations were initiated.⁽¹⁾ Investigations started during 1968 were continued during 1969. These include studies of the comparative productivity of a strain of hamsters from England and another strain from the United States; comparative productivity on pelleted diets containing either 10% fat, 4% fat, or 4% fat supplemented with apple three times a week; and comparative productivity of Syrian ham-

sters on granulated cellulose bedding and on white pine shavings. New studies initiated during 1969 include a comparison of productivity at 65°, 73°, and 80° F, and a comparison of productivity under the usual cool-white fluorescent light sources and the recently developed full-spectrum fluorescent lamps.

A modified test mating system used for animals in all studies consists of placing the female in the male's cage for a seven-day period. The female is then separated and observed for pregnancy. If pregnant, she is not rebred until her litter is weaned. If she is not pregnant after two weeks, she is placed with the male again for another seven days.

Parameters used to determine productivity include the number of seven-day mating periods required to produce one fertile mating, the average number born and weaned per litter, the weaning percent, and the number of young weaned per female during her entire reproductive life.

PROGRESS REPORT

The results to date are given in Tables 22 to 27.

Strain

Table 22 compares the total productivity of the American and English strains. The American strain is superior to the English strain in every category except its weaning percent. The total number of females observed in each strain included equal proportions of the two strains on the two different types of bedding and at the three different ambient temperatures. There is a slight disparity in the diets given. Eighty-

TABLE 22. COMPARATIVE PRODUCTIVITY OF AMERICAN AND ENGLISH STRAIN SYRIAN HAMSTERS

Strain	Number of females observed	Matings required for each fertile mating	Average number born	Average number weaned	Weaning percent	Number weaned per female
American	81	1.88	6.797	4.076	60	14.64
English	148	2.07	5.376	3.647	68	13.18

TABLE 23. COMPARATIVE PRODUCTIVITY ON 10% AND 4% FAT DIETS (BOTH AMERICAN AND ENGLISH STRAIN HAMSTERS)

Diet	Number of females observed	Matings required for each fertile mating	Average number born	Average number weaned	Weaning percent	Number weaned per female
10% fat	52	1.87	6.628	4.81	72	18.4
4% fat	23	2.66	6.67	4.84	73	14.7

TABLE 24. COMPARATIVE PRODUCTIVITY ON 4% FAT, 4% FAT, AND 4% FAT + APPLES DIETS (ONLY ENGLISH STRAIN HAMSTERS)

Diet	Number of females observed	Matings required for each fertile mating	Average number born	Average number weaned	Weaning percent	Number weaned per female
10% fat	22	1.7	6.34	4.73	75	19.4
4% fat	10	4.3	5.68	3.6	64	6.9
4% fat + apples	27	2.26	5.77	4.28	73	17.4

TABLE 25. COMPARATIVE PRODUCTIVITY WITH GRANULATED CELLULOSE AND WHITE PINE SHAVINGS BEDDING

Bedding	Number of females observed	Matings required for each fertile mating	Average number born	Average number weaned	Weaning percent	Number weaned per female
Granulated cellulose	20	2.0	6.68	4.67	70	16.1
White pine shavings	22	2.0	6.43	4.72	75	17.2

TABLE 26. COMPARATIVE PRODUCTIVITY AT THREE DIFFERENT AMBIENT TEMPERATURES

Temperature	Number of females observed	Matings required for each fertile mating	Average number born	Average number weaned	Weaning percent	Number weaned per female
65°F	64	1.88	5.84	2.70	46	9.70
73°F	102	2.13	6.38	4.66	73	17.32
80°F	63	1.90	5.01	3.45	69	11.9

TABLE 27. COMPARATIVE PRODUCTIVITY WITH DIFFERENT LIGHT SOURCES

Light source	Number of females observed	Matings required for each fertile mating	Average number born	Average number weaned	Weaning percent	Number weaned per female
Cool white fluorescent	15	8.2	1.7	4.0	85	2.2
Full spectrum fluorescent	50	6.0	4.92	2.4	49	1.80

four percent of the American strain received the 10% fat diet and the remainder received the 4% fat diet. Seventy-five percent of the English females received the 10% fat diet, 7% received the 4% fat diet, and the remaining 18% received 4% fat and apples. All females in both strains were kept under a cool white fluorescent light source.

Diet

Tables 23 and 24 show the diet comparisons. All animals were maintained at 73° F, on both types of bedding, and under cool white fluorescent lights. Table 23 contains data from both American and English strains, while Table 24 contains data only from the English strain. The two tables, therefore, cannot be compared directly. The 10% fat diet appears to be the most desirable, followed by the 4% fat and apples

and then the 4% fat. The data for the 4% fat groups in both tables may be misleading, however, because both tables include the 10 females listed in Table 24 which gave data entirely out of line from all other.

Bedding

A comparison of the two types of bedding (Table 25) shows slightly better reproductive performance on white pine shavings. The number observed, however, is relatively small. All animals were kept at 73° F under cool white fluorescent light and were given a 10% fat diet. The American and English strains were equally distributed.

Temperature

Table 26 shows distinct differences among the three ambient temperatures. Highest production is at 73° F, and lowest production is at 65° F. Postnatal loss was extremely high (54%) at 65° F. American and English strain hamsters were distributed in relatively equal proportions in each temperature group. All those at both 65° F and 80° F were given a 10% fat diet and were kept on granulated cellulose bedding. All three diets and both types of bedding were used with hamsters at 73° F. All were kept under cool white fluorescent lamps.

Light

Table 27 gives preliminary data on animals housed under different light sources. All hamsters were housed at 73° F, given 4% fat diet, and kept on granulated cellulose bedding. The initial groups gave very poor results with both light sources, and little can be concluded from the data obtained.

CONCLUSIONS

From the results obtained, there appears to be a genetic difference in productivity between the two strains of Syrian hamsters. It also appears that an ambient temperature of 73° F and a diet containing 10% fat are nearest to optimum, but there is little difference between productivity on granulated cellulose and white pine shavings bedding.

The results using the different light sources are inconclusive, and these studies are continuing. Studies on the effects of other diets, such as pellets containing 10% fat supplemented with apples are being made.

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A COAT COLOR MUTATION IN THE SYRIAN HAMSTER

Ronald W. Camden, and John E. Van Boskirk

PURPOSE AND METHODS

The purpose of this study is to define the phenotypic manifestations of a coat color mutation which appeared spontaneously in our colony of golden Syrian hamsters, English strain⁽¹⁾ and to determine the mode of genetic transmission. The phenotypic manifestations are needed to determine if there is any value in maintaining this mutant as a research animal. The mode of genetic transmission is needed to establish a breeding colony, if this is deemed desirable, and to eliminate the mutation from our golden colony.

The phenotypic manifestations are being determined by studying the pattern of pigment distribution and type of pigment in the hair and skin, both grossly and microscopically, and by comparing this to similar information for the normal golden hamster. The effects of the mutation on other phenotypic characteristics such as eye color, body size, and neonatal

survival is also being studied and compared with those of normal golden hamsters.

The genetic transmission is being determined in several ways. The number of mutant and normal animals and the sex ratios of each are being recorded for all litters in which the mutation appears. Mutant offspring from normal parents are being crossed and, if possible, a colony of pure breeding mutants will be established. Pure breeding mutants will then be crossed with normal golden and partial albino (another coat color mutation maintained in the Division) animals to determine allelism and involvement of various loci. The findings will then be compared to those reported in the literature for other coat color mutants and, if possible, matings will be made with phenotypically similar animals to check for allelism.

An estimate of the probable extent of the mutation in our present golden colony is also being made. This is being done by making a systematic search of pedigrees of golden hamster matings which produce mu-

tant progeny to determine if any common ancestor or ancestors exist.

PROGRESS REPORT

The pattern of pigment distribution in the mutant hair coat appears to be similar to that of the normal golden hamster with an obvious line of demarcation between the dorsal and ventral coat. The pigmentation of the ventral coat, where phacomelanin predominates, is grossly the same in mutant and normal animals. The dorsal coat, from the flanks and lower chest on up over the back, is much lighter in the mutant. The gross difference appears to be lack of eumelanin in the guard hairs of the mutant with the underfur remaining the same as in the golden. No microscopic studies have been completed.

The pigment distribution in the skin of the mutant animals is grossly altered from that in the golden. The ears and scrotum of the mutants are light in color owing to an apparent lack of eumelanin. No microscopic observations have been completed.

The eye color, body size, and neonatal mortality among mutants appear similar to those of the golden, but both the average litter size and average numbers weaned per litter are lower for the mutants than for the golden strain: 4.9 as compared to 5.4 born and 3.2 as compared to 3.6 weaned.

Twenty-one mutant animals with thirty normal golden sibs have been produced by normal golden parents. The sex ratio is 11 males to 10 females. Crosses of mutant animals have produced only mutant offspring, and a colony of pure-breeding mutants has been established. Ten litters from mutant parents have produced 15 males and 17 females. No crosses have been made between mutant animals and either normal golden or partial albino animals.

A search of the literature reveals numerous other coat color mutations in the Syrian hamster.⁽²⁾ From available descriptions, it appears the present mutation may be a recurrence of the autosomal recessive mutation cinnamon or brown (*b*). No animals from

stock in which this mutation occurs have been obtained for crossing with our mutants.

Ancestral pedigrees of matings producing mutant offspring have revealed the mutant was apparently present in at least three of the 22 pairs of hamsters used to initiate our English golden colony. None of these original pairs produced mutant animals in their immediate progeny. Of 161 pairs currently in breeding in the English golden colony, 148 trace their ancestry to one or more of the three carrier pairs.

CONCLUSIONS

This coat color mutation in the Syrian hamster appears to be the same as the previously reported autosomal recessive brown (*b*). The data we have obtained are compatible with autosomal recessive inheritance.

We have no indication that this mutant provides a useful research model. We are, however, maintaining a pure breeding colony of mutant animals in order to carry out further studies.

The mutant is probably widespread in our golden hamster breeding colony. There is no indication that the presence of the mutant in the heterozygous state in a phenotypically golden animal is detrimental to the research usefulness of that animal. It would, nevertheless, seem desirable to eliminate the mutant from the golden breeding colony. At present we are attempting to use as replacements only progeny of the 13 pairs not tracing their ancestry to one or more of the three known carriers. Because of other criteria for selecting breeder replacements, such as reproductive performance, we may need to use a system of test mating to mutants to select noncarrier replacements from progeny of the other 148 pairs of breeders.

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MONOGAMOUS MATING OF CHINESE HAMSTERS

Ronald W. Camden, Calvin M. Poole, and Robert J. Flynn

PURPOSE AND METHODS

Present methods of producing Chinese hamsters require large amounts of time, labor, and space. This study investigates the production of Chinese hamsters

using routine amounts of time, labor, and space.⁽¹⁾ We do this with a monogamous mating system by placing a male and female together for their complete reproductive life under conditions of controlled temperature, lighting, air exchange, and diet, similar to

those used in our mouse, rat, and other rodent-breeding colonies.

The successful use of monogamous matings for Chinese hamster production has been reported recently by others, but with a small number of breeders and with special procedures, including daily handling of animals until they became docile and supplementation of their diet with crushed oats, flaked maize, millet, sunflower seeds, and fresh apple.⁽²⁾ We are attempting to increase production and decrease aggressive tendencies and cannibalism by selection alone, and we are not supplementing the diet. Both sibling and nonsibling matings are employed, and we record the number of matings (breeding pairs), the percent of fertile matings, average litter size, average number weaned per fertile mating, and the percent adult mortality.

PROGRESS REPORT

A large number of nonsibling matings and five generations of sibling matings have been completed. Table 28 shows the total number of matings, the percent of matings fertile, the average weaned-litter size, and the average number of hamsters weaned per fertile mating. Also shown is the percent matings in which one adult (usually the male) was killed.

The percent of fertile matings declined to a low in the F₂ sibling matings but has increased in the later generations. Eight of the 19 F₅ sibling matings have not yet produced a litter, but are in breeding status. Later production by these matings will increase the percent of fertile matings for the F₅ siblings.

The average weaned litter size is lower for all generations of sibling matings than for nonsibling matings. Average litter size at birth was significantly lower than nonsibling matings only with the F₄ and F₅ sibling matings. The increase in postnatal mortality with the sibling matings reflects a relative increase in losses of entire litters. The mode litter size at weaning is 4 for all types of mating, except F₂ sibling matings, where the mode is 5.

The average number weaned per fertile mating is highest for the nonsibling matings. This number reflects both litter size and average number of litters per breeding pair. Because some pairs are still in breeding status, especially in the F₄ and F₅ groups, the

TABLE 28. MONOGAMOUS MATINGS MADE, PERCENT OF MATINGS FERTILE, AVERAGE LITTER SIZE, AND AVERAGE NUMBER WEANED PER FERTILE MATING.

Also Shown is the Percent of Matings in which one Adult (Usually Male) was Killed

Type of mating	Number of matings (breeding pairs)	Percent of matings fertile (at least one litter born)	Average litter size		Average number weaned per fertile mating (breeding pair)	Percent adult mortality
			Born	Weaned		
Nonsibling	139	50	4.39	3.57	11.9	35
F ₁ sibling	151	46	4.39	3.24	9.26	21
F ₂ sibling	89	35	4.59	3.07	7.03	21
F ₃ sibling	26	58	4.35	3.55	9.47	15
F ₄ sibling	21	71	4.09	3.35	7.60	29
F ₅ sibling	19	37	3.5	3.1	3.6	5

number of hamsters weaned per fertile pair will increase as more litters are weaned.

The percent of matings in which one adult (usually the male) was killed by its mate is highest in the nonsibling and has generally decreased with each successive generation of sibling mating. This gives an indication of the overall aggressive tendencies of adult Chinese hamsters toward their own species. The female is usually the most aggressive and kills the male, although we have a few examples of lethally aggressive males.

CONCLUSIONS

The decrease of aggressiveness through inbreeding and selection, as evidenced by the relative decrease in numbers of adults killed, is encouraging, but a decrease in the average litter size weaned is discouraging. This decrease is due to losses of entire litters prior to weaning and not to weaning of a large number of small litters or a decrease in average number born.

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FREQUENCY OF CAGE CLEANING AND THE SURVIVAL OF MICE

Robert J. Flynn and Calvin M. Poole

PURPOSE AND METHODS

This study was initiated to determine if the frequency of cage cleaning influences the health and survival of laboratory mice.⁽¹⁾

CF#1 Anl[Anl 66] female mice were divided at weaning into four groups of 245 mice each and two groups of 210 mice each. The cages of the first four groups are cleaned three times a week, twice a week, once a week, or once every 2 weeks. Cages of the other two groups are cleaned once every 4 weeks or are never cleaned. Spontaneous deaths are recorded daily.

PROGRESS REPORT

Until the mice were 6 to 8 months of age, no significant difference in their survival was noted.⁽¹⁾ During the next month, when the mice were 7 to 9 months of age, several died.⁽²⁾ While these deaths were thought to be due to *Pasteurella pneumotropica*,

this was not confirmed. Most mice died in cages that were cleaned least frequently (every four weeks or never).

The mice are now between 19 and 21 months of age. Although during the past year no epizootics were recognized, the mortality has increased with the increased age of the mice (Table 29). Mice in the cages that were never cleaned have the highest mortality. The differences in all the other groups are slight, with the mice in cages cleaned 3 times a week having the highest mortality.

CONCLUSIONS

From the results obtained to date, it appears that the frequency of cage cleaning has little effect on the health of young mice, unless an infectious pathogen is present. When this happens, decreased frequency of cage cleaning seems to result in increased mortality. When the mice get older, poor sanitation, associated with *never cleaning* the cages, appears to shorten the life-span; too frequent cleaning also may be detrimental.

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TABLE 29. MORTALITY OF MICE (AT 19-21 MONTHS OF AGE) RELATED TO FREQUENCY OF CAGE CLEANING

Frequency of cage cleaning					
Three times week	Two times week	Once week	Every 2 weeks	Every 4 weeks	Never
92/245 ^a	82/245	78/245	84/245	71/210	110/210
37.5%	33.4%	31.8%	34.2%	33.8%	52.3%

^a Number of deaths/total number mice.

HEPATIC AND RENAL LESIONS IN A HAMSTER BREEDING COLONY

Thomas E. Fritz, Patricia C. Brennan, David V. Tolle, and Robert J. Flynn

PURPOSE AND METHODS

Poor reproductive performance in a Syrian hamster breeding colony stimulated the examination of culled breeders and selected stock animals to determine disease incidence and its probable causes.⁽¹⁾

PROGRESS REPORT

In last year's report we described the results of pathologic, microbiologic, and toxicologic examinations conducted on animals from our breeding colonies.⁽¹⁾ With liver and kidney lesions the most promi-

nent pathologic findings. No bacteria of pathogenic significance were isolated nor were any toxic materials found in food or bedding specimens examined by independent laboratories.

A cell-free filtrate and a cell suspension prepared from livers and kidneys from three affected animals were inoculated intraperitoneally into litters of 2-day-old hamsters obtained from an outside source. A third litter, which served as a control, was inoculated only with the diluent (Eagle's basal tissue culture medium containing 200 units of penicillin and 100 µg

streptomycin ml). A fourth litter served as uninoculated controls. Four litters of day-old CF#1, Anl[Anl 66] mice were inoculated similarly—one litter each was inoculated with the filtrate, cell suspension, and diluent, while the fourth litter served as uninoculated controls.

At 3 months, 6 months and 13 months of age, at least two animals from each group of mice and hamsters were killed and necropsied. No gross lesions were present in any of the animals, nor were any microscopic lesions observed in hematoxylin and eosin stained sections of liver and kidney prepared by the paraffin method.

During the past year, there have been no additional efforts to collect data on pathologic lesions in recently killed hamsters. There have been no illnesses, other

than traumatic lesions from fighting and a few cases of diarrhea, and no hepatic or renal lesions have been observed.

The cause(s) of the liver and kidney lesions described in our previous report remain unidentified; animal inoculations have failed to reproduce the disease. Adequate reproductive performance and the absence of hepatic and renal lesions during the past year have caused us to abandon our search for the cause of the lesions.

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THROAT FLORA OF A CLOSED COLONY OF BEAGLE DOGS

Patricia C. Brennan and Richard C. Simkins

PURPOSE AND METHODS

In the 1968 Annual Report, we presented preliminary data on the throat flora of 165 dogs in the purebred beagle colony in the Division of Biological and Medical Research.⁽¹⁾ We continued and expanded this survey in 1969 and now have results for a one-year period. Four hundred sixty-seven dogs have been cultured.

Throat swabs were collected as before by swabbing the left tonsillar area of each dog when it received its routine physical examination. In addition to the media used previously (blood agar plates, PPLO agar plates, and trypticase soy broth), phenylethyl alcohol agar for gram-positive cocci, Rogosa agar for lactobacilli, Chapman Stone agar for staphylococci, eosin methylene blue agar for coliforms and Sabarauds, mycophil and mycosel agar for fungi also were inoculated. The media for fungi were incubated at 25° C, and all others were incubated at 37° C. Individual colonies of different types were picked from each medium and identified by routine biochemical and serologic methods.

PROGRESS REPORT

The kinds of bacteria and their incidence are presented in Figure 53. Alpha streptococci, nonhemolytic streptococci, *Neisseria* species, *Corynebacterium* species and *Bacillus* species are regarded as normal inhabitants of the throats of dogs and man.⁽²⁾ *Mycoplasma* species were present in all the dogs cultured.

Serologic tests showed that these strains are unrelated to *M. canis*, *M. muculosum*, and *M. spumans*, the common canine strains. Beta hemolytic streptococci were carried by 41.8% of the dogs, and all that were tested were Lancefield group G. We found an incidence of almost 80% *Pasteurella multocida*, in contrast to our earlier report of 45%.⁽¹⁾ *Diplococcus pneumoniae* and *Hemophilus* species were not isolated.

Because of the relatively high incidence of β hemolytic streptococci in the colony and because of its interest to us as a cause of bacteremia in irradiated dogs,^(3,4) we studied the incidence by age, sex, strain, kennel location, and month in which the culture was obtained.

Younger dogs, 1 to 4 years of age, had a significantly lower incidence than dogs 4 years old and older. The incidence of β hemolytic streptococci was similar for all months except October and November, when an extremely low (20%) incidence was noted. Analysis of the incidence by kennel location showed that the kennels housing breeding adults and stock dogs have a lower incidence than those in which experimental or control dogs are housed. Stock dogs are younger than either the experimental or control dogs and, thus, the differences noted are probably related to age rather than to environment. No sex differences were noted in the incidence, nor were differences observed between the two strains of beagle dogs in the colony.

FIG. 53.- Kinds of bacteria recovered from throat swabs and their incidence in a closed colony of beagle dogs

CONCLUSION

Our results indicate that the throat flora of dogs is generally stable, even when different methods are used to isolate bacteria and even when the dogs are subjected to different environmental conditions. Such stability of the flora was suggested before by Clapper and Meade⁽²⁾ from their experience with 25 dogs.

The significance of the high incidence of *Mycoplasma* species is not clear. Edward and Fitzgerald⁽⁵⁾ found a high incidence of *Mycoplasma* species in both the throat and vagina associated with infertility in dogs. Possibly *Mycoplasma* species contribute to the low conception rate observed in our dog colony,⁽⁶⁾ but this has not been determined.

The incidence of β hemolytic streptococci is of interest in terms of both its influence on radiation induced septicemia and on the overall health status of the dogs. Group G, β hemolytic streptococci have been associated with tonsillitis and with neonatal deaths in dogs^(7, 8) and, recently, with an epidemic of pharyngitis in man.⁽⁹⁾ Clearly, the group G, β hemolytic streptococci in the dogs in this colony must be regarded as pathogenic rather than as harmless commensals.

We know of no studies on the epidemiology of β hemolytic streptococci in a closed colony of dogs that allow direct comparisons with our results. Better comparisons will be possible when we have studied the same colony another year.

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MULTIFACTORIAL ANALYSIS AS A TOOL FOR THE STUDY OF EVOLUTIONARY AND ORGANIZATIONAL ASPECTS OF MAMMALIAN STRUCTURE AND FUNCTION

George A. Sacher

PURPOSE AND METHODS

Previous reports and publications have discussed the practical and theoretical reasons for our efforts to develop a comparative biology of longevity, aging, and radiation life shortening in mammals.^(1,2) The fundamental postulate underlying these efforts is that all mammalian species are variations on a single basic plan of organization. To the extent that this is true, the differences between species are expressible quantitatively as differences in the numerical values of a common set of system parameters. This postulate is methodologically valid, for it leads to the formulation of specific falsifiable hypotheses. It is important that the implications of the postulate be examined thoroughly, for a great deal of animal research on aging and late radiation effects is based on the implicit assumption that it holds. The research program at Argonne seeks to develop the kind of comparative biology of mammals that is needed if interspecies inferences are to be put on a scientific basis.

If all mammals are, by hypothesis, characterized by the possession of common system properties, then it becomes necessary to answer questions of the following form: how many distinct parameters are required to characterize the performance of a specified function by a particular species in the class? Two complementary approaches to these questions must be developed. One is the approach via the analysis of mechanisms. The other is the approach via the exhaustive description of the structures and functions concerned by appropriate techniques of multivariate analysis. The generic name for the latter approach is *factor analysis*.⁽³⁾

The basic postulate of factor analysis is that each measurable trait or performance of an individual can be represented as a weighted linear combination of the underlying contributory determiners, or *factors*. Each individual is characterized by a set of coefficients, or *weights*, for the set of factors. If individuals differ in the performances in question, then they must differ in their weights on some or all of the factors. The task of factor analysis is to determine how many factors are required to account for the differences in performance in a sample of individuals, and to esti-

mate the individual weights for those factors. The factor problem can be solved if it is possible to measure the scores on a sufficiently large set of related functions, and if these measurements can be carried out on a sufficiently large sample of individuals who vary significantly and independently in their performances of the functions. The inequality to be satisfied is that the total number of independent measurements (individuals $\times$ functions) must be several-fold larger than the total number of parameters to be estimated (individuals $\times$ factors). The number of individuals examined must also be absolutely large enough so that the sampling errors of the measures of association (covariances and correlations) are as small as necessary.

Factor analysis is restricted to postulating linear models because the general problem of multivariate nonlinear analysis is still intractable. If the linear model is accepted, then the relations between a set of n variates are completely contained in the $n \times n$ matrix of covariances or correlations, and the problem of discovering the factorial structure of the set of variates can be dealt with through the analysis of the matrix.

The representation of a matrix in terms of a set of *orthogonal* factors is effected by means of a classical procedure of matrix algebra known as *diagonalization*, which consists of performing a rigid rotation that transforms the original matrix into an equivalent matrix for which all the off-diagonal entries are zero. The diagonal entries are the *eigenvalues*, which are the variances of the newly-formed factors. Each factor has a row of numbers, known as its *eigenvector*, which specifies the factor in terms of the weight of the contribution of the original variates to it.

Other important algebraic manipulations are performed before the final set of acceptable factors is determined and the analysis reaches a stopping point. The necessity for these steps arises from the fact that there are an infinite number of equivalent orthogonal factor descriptions of a given covariance matrix. The particular one found by the diagonalization procedure has advantages as a starting point, but it has zero probability of being the desired end point. The proc-

ess of finding a best set of reference coordinates is dealt with by a process known as *rotation*. The purpose of rotation is to bring the factors into a configuration which satisfies certain conditions of meaningful interpretation, and to do so by completely objective procedures, which introduce no information about the substantive content of the variates or factors into

FIG. 54. Two factors found in the analysis of a 14×1 correlation matrix of brain and body measurements listed below. The 14 variates were measured on each of 63 species of insectivores (shrews, moles), prosimians (lemurs, galagos) and anthropoids (monkeys, apes, and man). The loadings of each variate on factor I are plotted on the abscissa vs. the loadings on factor II plotted as the ordinate. The dashed coordinates are the axes of the eigenvectors of the initial diagonalization; the solid coordinate axes are the axes found by varimax rotation.⁽¹⁾ Varimax factor I is interpreted as the *neocorticalization* factor because neocortex volume (variate 12) is the leading variate. Factor II is identified as the rhinencephalic, or paleocortical factor because olfactory bulb (variate 6) is the dominant variate. The angular relations between the variates are not altered by rotation. Three additional factors have been extracted from the 14×63 data matrix.

List of variates

No.	Name	No.	Name
1	Medulla oblongata	8	Septum
2	Cerebellum	9	Striatum
3	Mesencephalon	10	Schizocortex
4	Diencephalon	11	Hippocampus
5	Telencephalon	12	Neocortex
6	Bulbus olfactorius + bulbus accessorius	13	Brain weight
7	Paleocortex + amygdala	14	Body weight

Variates 1 through 12 are volumes of the indicated regions in mm³, variates 13 and 14 are weights in grams. All variates were converted to logarithms before the correlations were computed.

the process. By the nature of the process, rotation can only be partially successful, and the rotated factors must, therefore, be considered to be provisional and subject to further adjustment as more information, drawn from outside sources, is brought into the evaluation by the investigator. This is, however, an essential characteristic of all procedures for discovering meaning in natural phenomena. There is no branch of science in which truth emerges as the outcome of completely mechanical manipulations.

PROGRESS REPORT

Despite some very deep problems still not solved, factor analysis is enormously successful as a heuristic procedure. It has changed psychology, education, and vocational selection radically, and for the better. It is used extensively in social sciences and earth sciences, and is rapidly coming into use in biological sciences. The applications to be described here are new, but at the same time typical. They are listed and briefly described below.

Relation of Longevity of Mammalian Species to Brain Structure

Previous application of the system-theoretic approach had led to the discovery that life-span is highly correlated with brain weight, independent of body weight and metabolic rate.⁽¹⁾ The present study has produced a factorial description of brain structure in mammals, and has led to tentative identification of a specific brain factor associated with longevity.⁽⁴⁾ This is the factor for *neocorticalization*. Four other brain form factors have been isolated and tentatively identified (Figure 54). The progress of this work was greatly facilitated by the cooperation of Dr. Heinz Stephan, of the Max Planck Institute for Brain Research, Frankfurt, Germany. Future work will be done with his continued collaboration and in collaboration with other neuroanatomists and neurophysiologists.

Relation of Life-span to Hypophysis Size and Structure

No specifically hypophyseal factor was found: the hypophysis has a remarkably close allometric relation to body size.

Relation of Life-span to Metabolic Rate, Brain Weight, and Body Weight

Mammals have a wide range of homeothermic and heterothermic adaptations, so metabolic rate is not dependent on body size alone. Data on nine metabolic and related variates were tabulated for 89 mammalian species. Two metabolic factors were found. One relates to body size and overall metabolic rate and

the other to the thermal conductivity parameter. Life-span has relation only to the body size parameter.

Relation of Gestation Time to Brain and Body Weight at Birth, and to the Factors of Increase of These from Birth to Maturity

This study shows that gestation time and, by implication, time to sexual maturity, is a most important limiting factor in the evolution of large-brainedness, for the duration of the developmental periods is closely related to neonatal and adult brain weight, and this relation holds uniformly for the whole class Mammalia. The relation of these development periods to body weight is significant also, but considerably weaker.

CONCLUSION

As a result of these studies, we have attained, within a comparatively short time, a better qualita-

tive and quantitative conception of some important characteristics of mammals *qua* mammals. Cognate studies are in view on the factors identifiable within the family Muridae, and these will be relevant to our current and projected investigations of the comparative biology of radiation life shortening, which are being carried out with murid rodents.

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COMPUTER SIMULATION OF PERCENT LABELED MITOSES CURVES

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PURPOSE AND METHODS

A recent paper⁽¹⁾ provides complete details on our theoretical model describing "percent labeled mitoses" curves (PLM-curves). It also explains why it is important to investigate from a theoretical point of view this method of cell cycle analysis, so frequently used in the laboratory. The main result in Reference 1 is an equation for the Laplace transform of the PLM-curve. In simple cases, the transform can be inverted explicitly to give the PLM-curve as a function of time, $P(t)$. Thus, we have been able to rederive an equation previously obtained by Takahashi,⁽²⁾ and to correct an error in the work of Barrett.⁽³⁾ Furthermore, an algorithm for computer simulation of PLM-curves by a Monte Carlo technique is briefly outlined.

PROGRESS REPORT

A second paper⁽⁴⁾ analyzes the earlier work in greater detail. Two additional derivations are given for the main equation found in Reference 1. One of them, like Barrett's,⁽³⁾ based on renewal theory, and the second on the theory of branching processes. By a simple argument, we are led to a natural decomposition of the

PLM-curve into a sequence of "waves," $P(t) = \sum_{(v)} P_v(t)$, each wave being produced by cells of a single generation. The area under the v th wave, $\int_{t=0}^{\infty} P_v(t) dt$, is very nearly equal to the average duration of the S -period. According to Mendelsohn and Bresciani that area should be exactly equal to the mean DNA-synthesis time; our results provide some theoretical justification for this hypothesis.

A second computer program for simulating PLM-curves, similar to Barrett's, has also been developed.

An interesting by-product of our study^(4, 5) was the following. In a simple age-dependent branching process,⁽⁶⁾ characterized by a probability density $f(\tau)$ for the distribution of interdivision times, τ , the expected number of cells will eventually become proportional to e^{ct} , where t is time and c , the specific growth rate, is uniquely determined by the equation

$$2 \int_{\tau=0}^{\infty} e^{-c\tau} f(\tau) d\tau = 1. \quad (1)$$

It can be shown that there exists an upper bound for the solution, c , of Equation 1 if the density $f(\tau)$ has a sufficiently small coefficient of variation, v . In fact,

$$c \leq \frac{1}{D(1+v^2)} \ln \left(\frac{2}{1-v^2} \right), \quad (2)$$

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provided that $v < 1$. Here D is the mean generation time. Moreover, c is always $> (1/D) \ln 2$.

CONCLUSION

One of the principal authors (PJB) was on leave of absence for the past year, and therefore it is not certain at this point what direction future research on the PLM-process will take. A possible extension of the model would include correlations between the sojourn times of cells in the four phases of the life cycle (G_1 , S , G_2 , and M). These correlations are excluded at present, but they certainly exist in real systems.⁽⁷⁾ One could also study PLM-curves in cellular populations for which the number of cells (in particular the number of S cells) oscillates with a period of approximately 24 hr. Such circadian rhythms have been observed in some systems.

In any case, a third paper will be written to give full details on the two computer programs now available.

THE AREA RULE OF CELL KINETICS

Robert N. Buchal,* David L. Phillips,* and Ernesto Trucco

PURPOSE AND METHODS

In studying certain steady state biological systems, it is common to assume that the system can be represented as a set of connected compartments. Each compartment has at least one input and one output through which some material flows. Usually the material consists of cells, but in other cases it may consist of molecules.⁽¹⁾ For convenience, this material will be denoted by the term "cells."

The properties of a compartment can be investigated by introducing some cells labeled with a radioactive isotope (e.g., tritium, administered in the form of tritiated thymidine). At any given time and for each compartment, the labeling index, L , is defined as the ratio m/M , where m is the number of labeled cells and M the total number of cells in the compartment. Similarly, if N cells, of which n are labeled, enter the compartment per unit of time through one of the inputs, the ratio $p = n/N$ is the labeling index of that particular input. Labeling indices for all other inputs and outputs are defined in the same way. For a steady state system the quantities M and N will be constant, but in general m and n are functions of time, and therefore L and p also vary with time.

The simplest form of the area rule (which may be

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called the "fundamental area rule") deals with a single compartment satisfying the following conditions:

(a) The compartment as a whole is in a steady state (but, as noted above, there is a transient flow of labeled cells).

(b) The compartment has only one input and may have any number, $k \geq 1$, of outputs (e.g., if $k = 2$, one of the outputs could be cell death, and the other a flow of cells into a following compartment).

(c) There is no uptake of label within the compartment. In the case of tritiated thymidine (³HTdR), this applies to nonproliferative compartments which will acquire labeled cells only through inflow from proliferating precursors. It is also assumed that cells, once labeled, do not lose their label.

(d) Every cell has a finite, though possibly very long, sojourn time in the compartment.

(e) Initially (at time zero) there are no labeled cells in the compartment. Furthermore, the influx of labeled cells should tend to zero for $t \rightarrow \infty$, in such a way that the integrals of Equation (1) below have a finite value. Both of these conditions are met in nonproliferative compartments if the label used is ³HTdR and if it is given for a limited period of time (e.g., as a single pulse), because the number of cells labeled above background in the proliferative compartments will ultimately diminish to zero.

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(f) Biologically, labeled cells behave in exactly the same way as unlabeled cells. Thus, the probability that a labeled cell will leave the compartment through any one of the outputs should be the same as the corresponding probability for an unlabeled cell. Otherwise, that output would tend to favor either labeled or unlabeled cells, which is contrary to the assumption that the two types of cells are biologically indistinguishable.

Now, let $p(t)$ be the labeling index of the input flow and $p'_i(t)$ the labeling index of the i th output flow ($i = 1, 2, \dots, k$). If conditions (a)-(f) hold, it can easily be shown that

$$\begin{aligned} \int_{t=0}^{\infty} p(t)dt &= \int_{t=0}^{\infty} p'_1(t)dt = \int_{t=0}^{\infty} p'_2(t)dt \\ &= \dots = \int_{t=0}^{\infty} p'_k(t)dt, \end{aligned} \quad (1)$$

i.e., the total areas under the plots of the functions $p(t)$, $p'_i(t)$ vs. time must all be equal.

In practice, the quantities $p(t)$, $p'_i(t)$ are not easy to measure, but the labeling index, $L(t)$, can be obtained without much trouble. This is done by taking small samples from the compartment at frequent intervals and determining the ratio of labeled to unlabeled cells in each sample. Except for statistical fluctuations, that ratio will be the same as the labeling index of the whole compartment (assuming perfect mixing of labeled and unlabeled cells within the compartment). Clearly, then, the measurement of $L(t)$ is equivalent to an additional output that will not appreciably disturb the steady state situation. Therefore, Equation (1) can be extended as follows:

$$\begin{aligned} \int_{t=0}^{\infty} p(t)dt &= \int_{t=0}^{\infty} p'_i(t)dt = \int_{t=0}^{\infty} L(t)dt \\ (i &= 1, 2, \dots, k). \end{aligned} \quad (2)$$

Finally, suppose that there are two or more, say r , compartments satisfying the conditions listed above; let them be arranged in series with one and only one output from each compartment, except the last, serving as input to the following compartment. Equation (2) shows that

$$\int_{t=0}^{\infty} L_1(t)dt = \int_{t=0}^{\infty} L_2(t)dt = \dots = \int_{t=0}^{\infty} L_r(t)dt, \quad (3)$$

where $L_j(t)$ is the labeling index of the j th compartment ($j = 1, 2, \dots, r$).

PROGRESS REPORT

As a simple application of these results consider the curves published by Patt and Maloney⁽²⁾ for the labeling indices of nonproliferative compartments in the granulopoietic system. There are four such compart-

FIG. 55. Labeling index vs. time for metamyelocytes, band cells, and circulating granulocytes (from Patt and Maloney).⁽²⁾

ments: metamyelocytes, band cells, mature neutrophils in the marrow, and circulating cells; presumably they form a sequence of the type discussed in the last paragraph, so that each cell flows through them in succession.

The experiments were performed with dogs (beagles) to which a single injection of tritiated thymidine had been given. Figure 55, redrawn from Figures 2 and 3 of Reference 2, shows the functions $L(t)$ for three of the four compartments (metamyelocytes, band cells, and circulating granulocytes; the labeling index of mature cells in the marrow could not be measured). The areas under the three curves are as follows: metamyelocytes, 12.6 hr, band cells, 13.5 hr, and circulating cells, 23.2 hr.* These were obtained by straight line interpolation and straight line extrapolation from the last two points, which obviously introduces some error. Nevertheless, the area of the curve from the blood is nearly twice as large as that of the metamyelocytes or of the band cells, while according to Equation (3) all three areas should be equal. A discrepancy by a factor of two seems too large to be due to experimental error. It is thought that all the assumptions listed earlier are reasonably well fulfilled in the granulopoietic system, and a satisfactory explanation for this surprising area discrepancy has not yet been found.⁽³⁾

Making appropriate assumptions, the area rule can be extended to include compartments with more than one input and also, in some cases, proliferative compartments. Interesting results along these lines have been obtained, but they will not be reported here because complete details are given in a paper that is being published.⁽⁴⁾

Furthermore, there is a whole hierarchy of conservation laws that can be derived for the higher moments of the functions $p(t)$ and $p'_i(t)$. (The area, obviously, is the zeroth moment.) However, to be really useful in

* Notice that the integrals in Equations (1), (2), and (3) have the dimension of time because the labeling indices are pure numbers (with values ranging between zero and one).

practice these equations would require experimental data that are not available now with sufficient accuracy. The calculations are presented in Reference 5.

CONCLUSION

Conservation laws have a unique place in the understanding of models, and the area rule is no exception. For one thing, it can be used to test the consistency of a model system, as shown by the analysis of Patt and Maloney's labeling index curves. In other cases, the area rule provides simple derivations of results that could otherwise be obtained only by a rather complicated procedure. A typical example is its application to the system studied by Brues and Tyler⁽¹⁾ (of which a full discussion is given in Reference 4). Also notice that results such as Equation (3) are completely independent of many detailed features that may be specified for a particular system (e.g., the distributions of cellular sojourn times within each compartment).

It is likely, therefore, that the area rule will turn out to be a useful tool in the study of cellular kinetics.

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A NOTE ON HELMSTETTER AND COOPER'S MODEL FOR BACTERIAL REPLICATION AND ON RELATED WORK BY MARR, PAINTER, AND NILSON

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PURPOSE AND METHODS

Using *Escherichia coli* B, r, S. Cooper and C. E. Helmstetter⁽¹⁾ have proposed that cell division occurs at a fixed time, $C + D$, after each initiation of DNA replication. In rapidly growing cultures, when $C + D$ is larger than the interdivision time, τ , the bacterial chromosome will have multiple replication forks, at least for part of the cell's life cycle (for details see Reference 1). A. G. Marr et al.⁽²⁾ point out that the simple model of Cooper and Helmstetter ignores variability in the frequency of times between initiations, as well as variability of the time periods $C + D$. Then, they proceed to derive an equation for the probability density of interdivision times, $f(\tau)$ [Equation (14) of Reference 2]. A rigorous probabilistic treatment of the process described by Helmstetter and Cooper is difficult (see discussion below), but a reasonable first approximation follows: there is a (stationary) sequence of primary events, the initiations, each of which is followed by a secondary event (cell division) after a variable delay, $C + D$. It is required to find the probability density, $f(\tau)$, for the time intervals between secondary events. This kind of stochastic process has been discussed in some detail by M. ten Hoopen and H. A. Reuver.⁽³⁾

PROGRESS REPORT

Following Marr et al.,⁽²⁾ we denote by f_i the probability density function of interinitiation times and by f_d the probability density function of the delay times, $C + D$. We also assume that the two processes (initiations and delays) are statistically independent and, for simplicity, that the delay times, $C + D$, form a renewal process (Cox and Lewis, Reference 4, p. 78). Then, according to ten Hoopen and Reuver,⁽³⁾ the quantity $f(\tau)$ is given by

$$f(\tau) = \int_{\xi=0}^{\infty} f_i(\xi) g_1(\tau - \xi) d\xi, \quad (1)$$

where $g_1(\eta)$ is the probability density for the difference between two successive delays. Thus,

$$g_1(\eta) = \int_{x=-\infty}^{\infty} f_d(x) f_d(x + \eta) dx. \quad (2)$$

Inserting this expression into Equation (1) we obtain

$$f(\tau) = \int_{\xi=0}^{\infty} f_i(\xi) \int_{x=-\infty}^{\infty} f_d(x) f_d(x + \tau - \xi) dx d\xi. \quad (3)$$

Notice that in Equations (2) and (3) f_d may characterize either the distribution of delay times or the deviations of delays from their mean, in which case the

arguments of f_d may assume positive and negative values. For example, if f_d is a Gaussian density with mean zero (or any other fixed mean, $m \geq 0$) and a (sufficiently small) standard deviation σ , Equation (3) gives

$$f(\tau) = \int_{\xi=0}^{\infty} f_i(\xi) \frac{e^{-(\xi-\tau)^2/(2\sigma_0^2)}}{\sigma_0\sqrt{2\pi}} d\xi, \quad (4)$$

with $\sigma_0 = \sigma\sqrt{2}$.

In the following, however, we shall always take for f_d the probability density of the actual delays, so that $f_d(y) = 0$ for $y < 0$. Using this relation, and interchanging the order of the two integrations in Equation (3), we obtain

$$f(\tau) = \int_{x=0}^{\infty} f_d(x) \int_{\xi=0}^{x+\tau} f_i(\xi) f_d(x+\tau-\xi) d\xi dx. \quad (5)$$

This result should be compared with Marr et al.'s expression (which we denote by $f_M(\tau)$), namely,

$$f_M(\tau) = B \int_{x=0}^{\infty} e^{-kx} f_d(x) \cdot \int_{\xi=0}^{x+\tau} f_i(\xi) f_d(x+\tau-\xi) d\xi dx, \quad (6)$$

where k is the constant specific growth rate for the cellular population when in steady state of exponential growth, and $B (= G_0/N_0)$ is a constant > 1 such that

$$B \int_{x=0}^{\infty} e^{-kx} f_d(x) dx = 1. \quad (7)$$

We have no ready explanation for the discrepancy between Equations (5) and (6). Possibly, this arises from the fact that Marr et al., in deriving their results, had to consider a population in steady state of exponential growth. Therefore, $Be^{-kx}f_d(x)$ might well be the probability density of delay times for a sample taken from all extant cells in an exponentially growing population. However, it is not for such a sample that $f(\tau)$ is defined.⁽⁵⁾

In any case, both Equations (5) and (6) can only be approximately correct. This is seen most easily by computing the integral, $\int_{\tau=0}^{\infty} f_M(\tau) d\tau$, which turns out to be < 1 . In fact, the Laplace transform of $f_M(\tau)$ is given by

$$\begin{aligned} [Lf_M](s) &= B \int_{x=0}^{\infty} e^{-kx} f_d(x) \\ &\quad \cdot \int_{\tau=0}^{\infty} e^{-s\tau} \phi(x+\tau) d\tau dx \\ &= B \int_{x=0}^{\infty} e^{-kx} f_d(x) e^{-sx} \int_{u=x}^{\infty} e^{-su} \phi(u) du dx, \end{aligned} \quad (8)$$

where $\phi(u)$ denotes the convolution of f_i and f_d :

$$\phi(u) = \int_{\xi=0}^u f_i(\xi) f_d(u-\xi) d\xi. \quad (9)$$

If $s = 0$, the right hand side of Equation (8) is smaller than one. It follows from Equation (8), in particular, that Marr et al.'s Equation (15) for the variance of $f(\tau)$ is slightly incorrect.

Special cases of the inequality $\int_{\tau=0}^{\infty} f_M(\tau) d\tau < 1$ [or $\int_{\tau=0}^{\infty} f(\tau) d\tau < 1$] can easily be worked out in detail and are quite instructive. Suppose, for example, that in Equation (6) both f_i and f_d are negative exponential densities: $f_d(x) = \lambda e^{-\lambda x}$ and $f_i(\xi) = \mu e^{-\mu \xi}$, μ and λ being two positive constants. Then λ , B , and k must be related by the equation

$$\lambda B = \lambda + k \quad (10)$$

in order to satisfy (7), and one finds

$$\begin{aligned} f_M(\tau) &= \begin{cases} \frac{B\mu\lambda^2}{(\lambda - \mu)} \left[\frac{e^{-\mu\tau}}{(\mu + B\lambda)} - \frac{e^{-\lambda\tau}}{(B + 1)\lambda} \right] & \text{if } \lambda = \mu \\ \frac{\mu B}{(B + 1)^2} [1 + (B + 1)\mu\tau] e^{-\mu\tau} & \text{if } \lambda = \mu, \end{cases} \\ \int_{\tau=0}^{\infty} f_M(\tau) d\tau &= \frac{B[\mu + \lambda + B\lambda]}{(B + 1)(\mu + B\lambda)} \\ &= 1 - \frac{\mu}{(B + 1)(\mu + B\lambda)}. \end{aligned} \quad (11)$$

This last expression will be close to one, if μ/λ is sufficiently small. Similarly, integrating Equation (4) over τ from 0 to ∞ , we have

$$\begin{aligned} \int_{\tau=0}^{\infty} f(\tau) d\tau &= \int_{\xi=0}^{\infty} f_i(\xi) \int_{u=\xi}^{\infty} \\ &\quad \cdot \frac{e^{-u^2/(2\sigma_0^2)}}{\sigma_0\sqrt{2\pi}} du d\xi < 1, \end{aligned} \quad (12)$$

and $\int_{\tau=0}^{\infty} f(\tau) d\tau \approx 1$ if σ is small compared to the mean of f_i .

As a last example consider the case studied by McGill (quoted in Reference 3) who took a Dirac delta function for $f_i(x)$ and a negative exponential for $f_d(\xi)$. Thus, $f_i(x) = \delta(x - T)$ and $f_d(\xi) = \lambda e^{-\lambda \xi}$, where T and λ are positive constants. Equation (5) then gives $f(\tau) = (\lambda/2) \exp[-\lambda|T - \tau|]$, and $\int_{\tau=0}^{\infty} f(\tau) d\tau = 1 - (1/2) \exp(-\lambda T)$, which is nearly equal to 1 if λT is very large, i.e., if $1/\lambda \ll T$.

Generally speaking, the approximate nature of Equations (5) and (6) is due to the fact that we are restricted to *order-preserving* time delays, in the sense explained by ten Hoopen and Reuver.⁽³⁾ Conceivably, there might be a very long time delay after a first initiation and a very short subsequent time delay after the following initiation, so that the order in which the secondary events occur would be inverted with respect

FIG. 56.- Helmstetter and Cooper's model with $C' = 40$ min, $D = 20$ min, and $I = 25$ min. The numbers above each branch indicate its relative length, assuming that a branch point progresses at a constant rate along the chromosome. The number below each figure is the time in minutes before cell division.

to the sequence of primary events. Such a situation, of course, must be avoided. It is difficult to formulate this condition analytically, but a rough criterion is that the standard deviation of f_i , σ_d , should be small compared to the mean of f_i , m_i . [In Figure 13 of Reference 2 we find $\sigma_d = 0.095$ hr, $m_i = 0.76$ hr, and thus $\sigma_d/m_i = 0.125$].

The opposite extreme of delays that are not *order-preserving* has been studied by Govier and Lewis for the special case when the primary events occur at fixed time intervals (see pp. 204 ff. of Reference 4 and references given there). Obviously, their assumptions are not applicable for a generalization of the Helmstetter-Cooper model.

Finally, it should be pointed out that each delay, $C + D$, is itself composed of two parts, namely, C and D . This feature is also treated in the paper by ten Hoopen and Reuver (Reference 3, pp. 604-605).

CONCLUSIONS

In addition to being limited to order-preserving time delays, a further complication of the Helmstetter-Cooper model is that one "event" must actually occur almost simultaneously on different branches of a chromosome or possibly on two chromosomes. As an illustration, consider the ideal case in which there is no variability of interinitiation times, I , or of delays, C and D . Assume, as Helmstetter and Cooper did, that $C = 40$ min, $D = 20$ min, and take $I = \tau = 25$ min. The sequence of chromosome configurations during the cell's life-span is shown in Figure 56 (taken from Figure 1 of Reference 1). At birth, i.e., 25 min before the next division, the cell contains one chromosome with three branch points, 1, 2, and 3 (Figure 56a). Five minutes after birth (Figure 56b) point 1 has reached the end of the chromosome. Ten minutes later (Figure 56c) the cell contains two separate chromosomes, and four new initiations (4, 5, 6, and 7) are started. Finally, at division (Figure 56d) the cell has two chromosomes, each of them equal to that of Figure 56a.

If there is some variability in the sequence of events along each branch of a chromosome, it is no longer obvious exactly when cell division will occur. Clearly, a stochastic model, incorporating such features, would be extremely complicated.

R. H. Pritchard et al.⁽⁶⁾ and C. E. Helmstetter⁽⁷⁾ have proposed models for the control of initiation. Essentially, both of them assume that initiation starts when a certain substance within the cell reaches a critical amount or a critical concentration. We may postulate, then, that if this happens, four initiations, such as 4, 5, 6, and 7 in Figure 56c, will begin at the same time and thus can be regarded as a single event. If similar regulatory mechanisms are present for the delays (C and D), then the model discussed might be valid because for all practical purposes we would have a simple sequence of initiations (with variable interinitiation times), each of them being followed by a variable delay period between initiation and cell division. Further work will be necessary to establish if this is really the case.

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ON THE AVERAGE CELLULAR VOLUME IN SYNCHRONIZED CELL POPULATIONS

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PURPOSE AND METHODS

This investigation arose in connection with a study of Sinclair and Ross,⁽¹⁾ who were interested in the mean cell volume of a population that initially (at time zero) consists of N_0 newborn cells, all with the same volume, v_0 . It is assumed that cell division is determined only by the cell's age and not by its volume. The probability density function for the distribution of interdivision times, τ , is denoted by $f(\tau)$. It is further supposed that cell death is negligible, so the population size will increase according to the laws of a simple age-dependent branching process.⁽²⁾ Let $N(t)$ be the expected number of cells at time t and put $M(t) = N(t)/N_0$. The function $M(t)$ satisfies Equation (2) of Reference 2:

$$M(t) = \varphi(t) + 2\{M*f\}(t) \quad (1)$$

where

$$\varphi(t) = 1 - \int_{x=0}^t f(x)dx, \quad (2)$$

and $\{M*f\}(t)$, the convolution of the two functions M and f , is defined by

$$\{M*f\}(t) = \int_{x=0}^t f(x)M(t-x)dx. \quad (3)$$

The Neumann series solution of Equation (1) is as follows:

$$M(t) = \varphi(t) + \sum_{p=1}^{\infty} 2^p \varphi*f_p(t), \quad (4)$$

in which f_p stands for the p -fold convolution of the function f , i.e., $f_1(t) = f(t)$ and

$$f_{p+1}(t) = \{f*f_p\}(t) \quad \text{for } p = 1, 2, 3, \dots$$

Putting

$$F_p(t) = \int_{x=0}^t f_p(x)dx = \{1*f_p\}(t), \quad (5)$$

one sees that

$$\{f*F_p\} = \{f*1*f_p\} = \{1*f_{p+1}\} = F_{p+1} \quad (6)$$

and furthermore, since

$$\begin{aligned} \varphi(t) &= 1 - \{1*f\}(t), \\ \{f*F_p\} &= \{1*f_p\} - \{1*f_{p+1}\} = F_p - F_{p+1}. \end{aligned} \quad (7)$$

Hence, Equation (4) can be written in the form

$$M(t) = 1 + \sum_{p=1}^{\infty} 2^{p-1} F_p(t), \quad (8)$$

from which it follows that

$$\dot{M}(t) = \sum_{p=1}^{\infty} 2^{p-1} f_p(t), \quad (9)$$

where $\dot{M}(t) = dM(t)/dt$. Also notice that in this model every cell disappearing from the population at mitosis is immediately replaced by two new cells of age zero and, therefore, the birth rate, $\alpha(t)$, must be equal to twice the net increase in number of cells:

$$\alpha(t) = 2N_0\dot{M}(t). \quad (10)$$

Sinclair and Ross⁽¹⁾ used a special form of Equation (8). Let $\bar{\tau}$ be the average generation time (assumed to be finite):

$$\bar{\tau} = \int_{x=0}^{\infty} xf(x)dx. \quad (11)$$

Suppose that the probability density $f(\tau)$ has a relatively small coefficient of variation and that the time, t , is not too large, so that no appreciable overlapping of generations occurs. Consider values of the variable t restricted to a sufficiently small neighborhood of $s\bar{\tau}$, where s is a positive integer. Then, in Equation (8),

$$F_p(t) \approx 1 \quad \text{for } p < s, \quad F_p(t) \approx 0 \quad \text{for } p > s,$$

and

$$M(t) \approx 1 + \sum_{p=1}^{s-1} 2^{p-1} + 2^{-1}F_s(t) = 2^{-1}[1 + F_s(t)],$$

or, using the relation $F_s(s\bar{\tau}) = 1$,

$$N(t) \approx N_0 \cdot 2^{s-1} \left\{ 2 \int_{x=0}^t f_s(x)dx + \int_{x=t}^{\infty} f_s(x)dx \right\}, \quad (12)$$

if $t \approx s\bar{\tau}$. This is Equation (2) of Reference 1.

Two further assumptions are made regarding cellular volume:

(a) The volume of each cell, v , is a given function of its age, a , and of its volume at birth, v_b :

$$v = \psi(a, v_b); \quad (13)$$

(b) When a cell divides, each of the two daughters has the same birth volume, equal to one-half that of the mother cell.

Special cases of Equation (13) considered by Sinclair and Ross are those of linear and exponential volume increase with age:

$$v = v_b + ra \quad (r = \text{constant} > 0), \quad (14)$$

and

$$v = v_b e^{ka} \quad (k = \text{constant} > 0). \quad (15)$$

Obviously, in Equation (4) the number of cells is expressed in terms of generations. $\varphi(t)$ represents the fraction of the original cells (zeroth generation) that have not yet divided at time, t . The quantity

$$R_\nu(t, a)da = N_0 2^\nu \varphi(a) f_\nu(t - a) da \quad (16)$$

is the expected number of cells present at time, t , belonging to the ν th generation ($\nu = 1, 2, 3, \dots$), which have ages between a and $a + da$. In fact, such cells will have undergone their ν th division at time, $t - a$, within da (or within dt , since $dt = da$); this occurs with probability $f_\nu(t - a)da$. Then, $\varphi(a)$ is the probability that a newborn cell survives to reach age, a , (the factor 2^ν accounts for cell doubling at each division).

The life history of ν th generation cell is specified by its ν interdivision times, $\tau_1, \tau_2, \dots, \tau_\nu$, and by its age, a . Alternatively, one may use a and the time periods

$$T_\lambda = \sum_{\mu=1}^{\lambda} \tau_\mu, \quad \lambda = 1, 2, \dots, \nu,$$

so that T_λ is the time at which the λ th division has occurred. These variables must satisfy the conditions

$$0 < T_\lambda < T_{\lambda+1} \quad \text{for } \lambda = 1, 2, \dots, (\nu - 1),$$

and $T_\nu = t - a$ (see Figure 57 for $\nu = 4$).

FIG. 57—Illustration of the meaning of the variables τ_i , T_i , a , and t .

Writing out the complete expression for the convolution integral, $f_\nu(t - a)$, in Equation (16), it becomes evident that the expected number of ν th generation cells present at time, t , which have age a (within da) and which, for $\lambda = 1, 2, \dots, (\nu - 1)$, have given values for the T_λ 's (within dT_λ) is equal to

$$N_0 2^\nu \varphi(a) [\pi_{\lambda-2}^p f(T_\lambda - T_{\lambda-1})] f(T_1) dT_1 dT_2 \dots dT_{\nu-1} da \quad (17)$$

$[\nu = 2, 3, 4, \dots],$

where $T_\nu = t - a$. If $\nu = 1$, this expression reduces to $2N_0 \varphi(a) f(t - a) da$. Now, using Equation (13) and halving the volume at each division, the volume of a ν th generation cell with a given life history can be expressed as a function of the quantities $v_0, T_1, T_2, \dots, T_{\nu-1}, t$, and a , say $\eta_\nu(v_0, t, a; T_1, T_2, \dots, T_{\nu-1})$. Therefore, the mean volume as a function of time, $\langle v(t) \rangle$, is obtained by multiplying the expression (17) with η_ν , integrating over $a, T_1, T_2, \dots, T_{\nu-1}$, summing over all generations, and finally dividing by $N(t)$. It is convenient to introduce $T_\nu = t - a$ as a new variable of integration instead of a . The resulting equation for $\langle v(t) \rangle$ is

$$\begin{aligned} \langle v(t) \rangle = \frac{1}{M(t)} & \left\{ \varphi(t) \psi(t, v_0) + 2 \int_{T_1=0}^t \varphi(t - T_1) f(T_1) h_1(v_0, t, T_1) dT_1 \right. \\ & + \sum_{\nu=2}^{\infty} 2^\nu \int_{T_\nu=0}^t \varphi(t - T_\nu) \int_{T_{\nu-1}=0}^{T_\nu} f(T_\nu - T_{\nu-1}) \int_{T_{\nu-2}=0}^{T_{\nu-1}} f(T_{\nu-1} - T_{\nu-2}) \\ & \dots \int_{T_3=0}^{T_4} f(T_4 - T_3) \int_{T_2=0}^{T_3} f(T_3 - T_2) \int_{T_1=0}^{T_2} f(T_2 - T_1) f(T_1) h_\nu(v_0, t; T_1, T_2, \dots, T_\nu) \\ & \left. dT_1 dT_2 \dots dT_{\nu-1} \right\}, \end{aligned} \quad (18)$$

in which h_v is the same as η_v with $t - T_v$ substituted for a .

To obtain the functions h_i , define the quantities v_i recursively by the equations

$$v_1 = {}^1_2\psi(\tau_1, v_0), \quad (19)$$

$$v_j = {}^1_2\psi(\tau_j, v_{j-1}) \quad \text{for } j \geq 2, \quad (20)$$

so that v_j is the cellular volume immediately after the j th division. For a v th generation cell present in the population at time $t = a + \sum_{\lambda=1}^v \tau_\lambda$, the volume will be $\psi(a, v_v)$. In this expression replace each τ_j by $T_j - T_{j-1}$ (with $T_0 = 0$) and a by $t - T_v$; this gives $h_v(v_0, t; T_1, T_2, \dots, T_v)$. In particular, for $v = 0, 1$, and 2:

$$h_0 = \psi(a, v_0) = \psi(t, v_0) \quad (21)$$

(time and age being the same for cells of the zeroth generation),

$$h_1 = \psi(a, {}^1_2\psi(\tau_1, v_0)) = \psi(t - T_1, {}^1_2\psi(T_1, v_0)), \quad (22)$$

$$0 < T_1 < t,$$

$$h_2 = \psi(t - T_2, {}^1_2\psi(T_2 - T_1, {}^1_2\psi(T_1, v_0))), \quad (23)$$

$$0 < T_2 < t, 0 < T_1 < T_2.$$

For example, in the case of linear volume increase with age [Equation (14)] one finds

$$v_v = \frac{v_0}{2^v} + \frac{r}{2^v} \sum_{\lambda=1}^v 2^{\lambda-1} \tau_\lambda$$

$$(v = 1, 2, 3, \dots)$$

and, hence,

$$h_0 = v_0 + rt,$$

$$h_v = \frac{v_0}{2^v} + rt - \frac{r}{2^v} \sum_{\lambda=1}^v 2^{\lambda-1} T_\lambda$$

$$(v = 1, 2, 3, \dots).$$

PROGRESS REPORT

Equation (18) can be written in a more compact form if the function $\psi(a, v_0)$ is of the "dispersionless" type, as defined by Anderson et al. (Reference 3, page 261). This means that

$$\psi(a, v_0) = v_0 g(a), \quad (25)$$

g being a function of age only, with $g(0) = 1$.

In that case, $h_0 = v_0 g(t)$, $h_1 = (v_0/2)g(T_1)g(t - T_1)$, and

$$h_v = \frac{v_0}{2^v} g(t - T_v)g(T_1)g(T_2 - T_1)$$

$$\dots g(T_v - T_{v-1}) \text{ for } v \geq 2.$$

Thus Equation (18) becomes

$$\langle v(t) \rangle = \frac{v_0}{M(t)} \left[\rho(t) + \sum_{v=1}^{\infty} \{\rho * m_v\}(t) \right], \quad (26)$$

where

$$\rho(t) = g(t)\varphi(t), \quad (27)$$

$$m(t) = g(t)f(t), \quad (28)$$

and m_v is the v -fold convolution of the function m .

The case of exponential volume increase with age [Equation (15)] is obtained by taking $g(a) = e^{ka}$ in Equation (25). This gives

$$h_v = \frac{v_0}{2^v} e^{kt}, \quad (29)$$

so that the volume of a v th generation cell is completely independent of its life history. From Equation (18) or (26) one then finds

$$\langle v(t) \rangle = \frac{v_0 e^{kt}}{M(t)} \left[\varphi(t) + \sum_{v=1}^{\infty} \{\varphi * f_v\}(t) \right].$$

Using Equation (7), however, it is seen that the square bracket in this expression has the value 1, and therefore

$$\langle v(t) \rangle = \frac{v_0 e^{kt}}{M(t)}, \quad (30)$$

if $\psi(a, v_0)$ is given by Equation (15).

Now it is known that for large values of t the function $M(t)$ becomes approximately equal to $M_0 e^{ct}$, where the specific growth rate, c , is uniquely determined by the relation

$$2 \int_{x=0}^{\infty} e^{-cx} f(x) dx = 1, \quad (31)$$

and

$$M_0 = \frac{1}{2c\vartheta} \quad (32)$$

with

$$\vartheta = 2 \int_{x=0}^{\infty} x e^{-cx} f(x) dx \quad (33)$$

[Equations (5) and (6) of Reference 2]. These results follow from Feller's theorems on the renewal equation.¹⁴ Therefore, if $t \rightarrow \infty$, the right hand side of Equation (30) tends to a finite limit, $\langle v(\infty) \rangle$, only if

$$c = k. \quad (34)$$

In fact, Bell and Anderson have shown¹⁵ that c must be equal to k ($\alpha = f_1$ in their notation) if there is no cell death and if the rate of cellular volume increase with age is proportional to volume itself.

However, even with $c = k$ there probably is instability, and one would expect that no steady state of balanced exponential growth will be achieved, as was pointed out by Bell.⁽⁶⁾ Therefore, if Equation (15) holds, it is unrealistic to postulate that cell division depends only on cellular age and not on the volume attained by the cell (this, too, is discussed by Bell⁽⁶⁾). More generally, for "dispersionless" volume increase with age [Equation (25)], and still assuming that the occurrence of mitosis is not influenced by cellular volume, it is likely that a steady state of balanced exponential growth will be reached, if at all, only for very special choices of the function $g(a)$.

Consider now the case of linear volume increase [Equation (14)]. From Equation (18) one can obtain a simple expression for the limiting value of $\langle v(t) \rangle$ as $t \rightarrow \infty$. It will be assumed that the function $f(t)$ is sufficiently well behaved, so that Feller's theorems⁽⁴⁾ are applicable, which is a very mild restriction. In particular, $f(\tau)$ must be regular at the origin [$F_1(0) = 0$]. [Actually, no cells divide at age zero, and so $f(0) = 0$.]

It can easily be shown that

$$\sum_{\lambda=1}^p 2^{\lambda-1} T_\lambda = (2^p - 1) T_1 + \sum_{\lambda=2}^p (2^p - 2^{\lambda-1}) (T_\lambda - T_{\lambda-1}) \quad (35)$$

($p = 2, 3, 4, \dots$).

Inserting the expression given by Equation (24) for h_p into Equation (18) and using Equation (35), one finds, since

$$\sum_{\lambda=1}^p (2^p - 2^{\lambda-1}) = 1 + (p-1)2^p, \\ \langle v(t) \rangle = \frac{1}{M(t)} \left\{ v_0 \left[\varphi(t) + \sum_{r=1}^{\infty} \{ \varphi * f_r \}(t) \right] + rtM(t) - r \{ \varphi * q \}(t) - r \sum_{p=2}^{\infty} [(1 + (p-1)2^p) \{ \varphi * f_{p-1} * q \}(t)] \right\};$$

here the function $q(t)$ is defined by

$$q(t) = t \cdot f(t). \quad (36)$$

With the help of Equations (6), (7), and (8) this becomes

$$\begin{aligned} \langle v(t) \rangle &= \frac{1}{M(t)} \left[v_0 + rtM(t) - r \sum_{p=1}^{\infty} p 2^{p-1} \{ F_{p-1} * q \}(t) \right] \\ &= \frac{1}{M(t)} [v_0 + rt + rS(t)], \end{aligned} \quad (37)$$

where

$$S(t) = \sum_{p=1}^{\infty} 2^{p-1} [tF_p(t) - p \{ F_{p-1} * q \}(t)] \quad (38)$$

and

$$F_0(t) = 1. \quad (39)$$

Now, putting

$$tF_p(t) = \gamma_p(t), \quad (40)$$

one has the simple identity

$$\begin{aligned} \int_{u=0}^t f(u)(t-u)F_p(t-u)du \\ = t \int_{u=0}^t f(u)F_p(t-u)du - \int_{u=0}^t u f(u)F_p(t-u)du \end{aligned}$$

or

$$\{f * \gamma_p\}(t) = tF_{p+1}(t) - \{q * F_p\}(t). \quad (41)$$

Therefore, convoluting both sides of Equation (38) with $2f(t)$:

$$\begin{aligned} \{2f * S\} &= \sum_{p=1}^{\infty} 2^p [\{f * \gamma_p\} - p \{q * F_p\}] \\ &= \sum_{p=1}^{\infty} 2^p [tF_{p+1} - (p+1) \{q * F_p\}] \\ &= S - [tF_1 - \{1 * q\}]. \end{aligned}$$

This shows that the function $S(t)$ satisfies the renewal equation

$$S(t) = B(t) + 2\{f * S\}(t) \quad (42)$$

with

$$B(t) = tF_1(t) - \int_{x=0}^t x f(x) dx = \int_{x=0}^t F_1(x) dx. \quad (43)$$

It follows then from Feller's theorem that for large values of t

$$S(t) \approx S_0 e^{ct}. \quad (44)$$

Here c is again determined by Equation (31) and

$$S_0 = \frac{\int_{t=0}^{\infty} e^{-ct} B(t) dt}{\vartheta} = \frac{1}{2c\vartheta}. \quad (45)$$

[Notice that for large values of t , $F_1(t) \approx 1$, $B(t) \approx t$, and the integral in Equation (45) converges.]

Consequently,

$$\lim_{t \rightarrow \infty} \left[\frac{S(t)}{M(t)} \right] = \frac{1}{c},$$

and so

$$\lim_{t \rightarrow \infty} \langle v(t) \rangle \equiv \langle v(\infty) \rangle = \frac{r}{c}. \quad (46)$$

This result is not unexpected because it can be shown^(5,7) that for a population in steady state of exponential growth the mean cellular volume is indeed equal to r/c if Equation (14) holds and if cell death is negligible.

The mean cellular volume at birth, $\langle v_b(t) \rangle$, can be calculated in much the same way as $\langle v(t) \rangle$. The total number of newborn cells (cells with age between 0 and dt) present in the population at time t is $\alpha(t)dt$, and the expected number of such cells belonging to the v th generation is obtained from Equation (16) by setting $a = 0$, $\varphi(0) = 1$. Cells of the zeroth generation have age > 0 if $t > 0$, and the function $\alpha(t)$ is given by Equation (10). Proceeding as in the derivation of Equation (18) one finds, for $t > 0$:

$$\begin{aligned} \langle v_b(t) \rangle = \frac{1}{M(t)} & \left\{ f(t)w_1(v_0, t) + \sum_{i=2}^{\infty} 2^{i-1} \int_{T_{i-1}-0}^t f(t - T_{i-1}) \int_{T_{i-2}-0}^{T_{i-1}-1} f(T_{i-1} - T_{i-2}) \int_{T_{i-3}-0}^{T_{i-2}-2} f(T_{i-2} - T_{i-3}) \right. \\ & \left. \cdots \int_{T_3-0}^{T_4} f(T_4 - T_3) \int_{T_2-0}^{T_3} f(T_3 - T_2) \int_{T_1-0}^{T_2} f(T_2 - T_1) f(T_1) w_i(v_0, t; T_1, T_2, \dots, T_{i-1}) dT_1 dT_2 \cdots dT_{i-1} \right\}, \end{aligned} \quad (47)$$

where $w_i(v) = w_i(v)$ is equal to h_i with T_i replaced by t .

For the exponential case [Equation (15)] this gives

$$\langle v_b(t) \rangle = \frac{v_0 e^{ct}}{2M(t)} G(t), \quad (48)$$

with

$$G(t) = \sum_{v=1}^{\infty} f_v(t). \quad (49)$$

The function $G(t)$ is a solution of the renewal equation

$$G(t) = f(t) + \{f * G\}(t)$$

and therefore tends to the finite limit $1/\bar{\tau}$ for $t \rightarrow \infty$. Differentiating both sides of Equation (1) it is found that

$$\dot{M}(t) = f(t) + 2\{f * \dot{M}\}(t),$$

and thus, for large t , $\dot{M}(t) \approx e^{ct} (2\vartheta) = cM_0 e^{ct}$.

In the linear case (Equation 14) one has

$$w_1 = \frac{v_0}{2} + \frac{r}{2}t,$$

$$w_v = \frac{v_0}{2} + \frac{rt}{2} - \frac{r}{2^v} \sum_{\lambda=1}^{v-1} 2^{\lambda-1} T_{\lambda} \quad \text{for } v = 2, 3, 4, \dots,$$

and it is easy to see that $\langle v_b(t) \rangle$ tends to a finite value, $\langle v_b(\infty) \rangle$, as $t \rightarrow \infty$. The calculations are very similar to those used in deriving Equation (46); the result is

$$\langle v_b(\infty) \rangle = 2r \int_{x=0}^{\infty} x e^{-cx} f(x) dx = r\vartheta. \quad (50)$$

Once again, Equation (50) can be obtained directly from Bell's theory for a cellular population in steady state of exponential growth.⁽¹⁰⁾ In the following, the notation of Reference 7 will be used, except that the quantity $\psi(v)$ of Reference 7 is denoted here by $\psi_1(v)$ to avoid confusion with the function ψ of Equation (13). Assume that there is no cell death and that cell volume increases linearly with age. Thus: $D(a, v) = 0$, $F(a, v) = r = \text{constant}$, $h(a, v) = v - ra$, $H(a, v) = 0$. Assume further that the function $P(a, v)$ of Reference 7 depends only on a but not on v : $P(a, v) = P(a)$ (with $\int_{x=0}^a P(x) dx \rightarrow \infty$ for $a \rightarrow \infty$). Then

$$f(a) = P(a) \exp \left[- \int_{x=0}^a P(x) dx \right],$$

and Equation (39) of Reference 7 becomes

$$\beta(a, v) = K \psi_1(v - ra) e^{-ca} f(a) P(a).$$

Inserting this expression into Equation (44) of Reference 7 gives

$$\psi_1(v) = 4 \int_{a=0}^{2v/r} \psi_1(2v - ra) e^{-ca} f(a) da.$$

Multiplying both sides of this equation with v , integrating over v , from $v = 0$ to $v = \infty$, and interchanging the order of the two integrations, one finds

$$\begin{aligned} \langle v_b(\infty) \rangle &= \int_{v=0}^{\infty} v \psi_1(v) dv \\ &= 4 \int_{a=0}^{\infty} e^{-ca} f(a) \int_{v=ra/2}^{\infty} v \psi_1(2v - ra) dv da \\ &= \int_{a=0}^{\infty} e^{-ca} f(a) \int_{u=0}^{\infty} (u + ra) \psi_1(u) du da \\ &= \frac{1}{2} \langle v_b(\infty) \rangle + \frac{r\vartheta}{2}, \end{aligned}$$

or $\langle v_b(\infty) \rangle = r\vartheta$.

Here the relations $\int_{a=0}^{\infty} e^{-ca} f(a) da = 1/2$ and $\int_{u=0}^{\infty} \psi_1(u) du = 1$ have been used.

The quantity ϑ is the first moment of the "carrier density" $2e^{-cx}f(x)$. It can be shown that, in steady state of exponential growth without cell death, ϑ is the average age at which the cells divide. Also, ϑ is slightly smaller than the doubling time, T , of the population [$T = (1/c) \ln 2$]. T itself is smaller than the

average generation time, τ . Thus: $\vartheta < T < \tau$ (Reference 8, page 469).*

Equation (50) says that, as $t \rightarrow \infty$, the mean volume at birth becomes equal to the volume increment during the time interval ϑ , a result which is intuitively very plausible. Notice that according to Equation (14) the average cell volume of the zeroth generation will be increased by approximately $r\tau$, which could be much more or much less than v_0 (of course, in real populations there may not be any cells with extremely large or extremely small volumes, but this is irrelevant for a discussion of the mathematical model). Subsequently, however, the mean volumes, $\langle v(t) \rangle$ and $\langle v_b(t) \rangle$, will gradually reach their asymptotic values. Equations (46) and (50) show that these limiting values do not depend on v_0 .†

The assumption that all the cells in the population have age zero initially is fulfilled with good approximation if the cells are synchronized by selecting those in mitosis. On the other hand, it will certainly not be true that every cell has the same initial volume, v_0 . If $\epsilon(v_0) dv_0$ is the fraction of cells with initial volumes between v_0 and $v_0 + dv_0$ ($\int_{v_0=0}^{\infty} \epsilon(v_0) dv_0 = 1$), the actual mean cellular volume will be

$$\langle \bar{v}(t) \rangle = \int_{v_0=0}^{\infty} \langle v(t) \rangle \epsilon(v_0) dv_0,$$

where $\langle v(t) \rangle$, which depends on v_0 , is given by Equation

* In Reference 8 the probability density of interdivision times is denoted by δ and the "carrier density" by f . Also, the quantities $\bar{l}$ and D of Reference 8 correspond, respectively, to ϑ and $\bar{\tau}$.

† It would appear from Sinclair and Ross' Equation 5 that they chose $r = v_0/\bar{\tau}$. In that case, of course, the right hand sides of Equations (46) and (50) would be proportional to v_0 : $\langle v(\infty) \rangle = v_0/c\bar{\tau}$ and $\langle v_b(\infty) \rangle = v_0\vartheta/\bar{\tau}$.

THE DISTRIBUTION OF CELLULAR VOLUMES IN EXPONENTIALLY GROWING POPULATIONS

Ernesto Truoco

PURPOSE AND METHODS

Much work has been done at this Laboratory on the volume spectrum of growing cell populations. One question of interest in these investigations was whether the volume increase of individual cells with age conforms more closely to the exponential or to the linear mode.⁽¹⁾ Recently, Kubitschek, who favors the latter view, has derived equations for the probability density of volume distribution, $\lambda(v)$, in the case of linear and of

exponential growth. For "dispersionless" growth, Equation (26) is still valid with v_0 replaced by $\bar{v}_0 = \int_{v_0=0}^{\infty} v_0 \epsilon(v_0) dv_0$.

CONCLUSION

The results derived here seem to differ from those of Sinclair and Ross, even though the basic assumptions are the same in both theories. It might be of interest to repeat Sinclair and Ross' calculations using Equation (18), with either (14) or (15), and see which of the two alternatives gives a closer fit to their excellent experimental curves. Perhaps an even better method would be to simulate the life histories of individual cells (including volume changes) with a Monte Carlo program and estimate the mean cellular volume by taking averages over sufficiently large numbers of cells.

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exponential single cell growth law (appendix of Reference 2). Sinclair and Ross⁽³⁾ state that they were unable to distinguish clearly between the two alternatives. On the other hand, Painter and Marr believe that "Most of the microscopic measurements of the growth of bacteria are consistent with the hypothesis of an exponential increase in volume with age." (Reference 4, page 533.)

Earlier studies along the same lines include the re-

markable paper by Scherbaum and Rasch,⁽¹⁵⁾ but it was only the subsequent improvement in Coulter counter techniques that made it possible to obtain accurate volume spectra.^(1, 3, 8-9)

For a cell population in steady state of exponential growth, the quantity $\lambda(v)$ can be calculated from the equation of Collins and Richmond,^(7, 10) provided that the volume distributions of newborn cells and of dividing cells are known. Collins and Richmond's result, in turn, may be obtained from the formalism developed by Bell^(11, 12) or from similar equations.⁽¹³⁾ For simplicity, the theory of Bell and Anderson will be used.

In Bell's treatment the state of a single cell is determined by two parameters, its age, a , and its volume, v . The density function for cells of age a and volume v is denoted by $n(t, a, v)$, meaning that $n(t, a, v) da dv$ represents the expected number of cells present at time, t , with ages in the range $(a, a + da)$ and volumes in $(v, v + dv)$. [The notation used here differs from that of Bell et al. who write $N(t, \tau, V)$ instead of $n(t, a, v)$.] Two further functions characterizing the cell population are $P(a, v)$ and $D(a, v)$, defined as follows: $n(t, a, v) P(a, v) \cdot dt da dv$ (or $n(t, a, v) D(a, v) dt da dv$) = expected number of cells dividing (or dying) during the time interval dt with ages in $(a, a + da)$ and volumes in $(v, v + dv)$.

It is also assumed that a dividing cell gives two daughters of exactly equal size, and that the volume of each cell, v , is a well defined function of its age, a , and of its volume at birth, v_0 . Thus, $v = b(a, v_0)$. The rate of increase of cell volume with age is $F(a, v)$, so that $F(a, v) = \partial b(a, v_0) / \partial a$, where, after differentiation, v_0 on the right side must be expressed in terms of a and v . The function $b(a, v_0)$ is taken to be strictly increasing with its two arguments, a and v_0 . This assumption is probably too restrictive in some cases (for cell volume may well remain stationary or even shrink over certain age ranges), but mathematically it is convenient because it makes the transformations of variables introduced below unambiguous. For the simple cases of linear and exponential volume increase with age, $b(a, v_0)$ is indeed a monotonic function of a and of v_0 .

More generally, one may think of the three variables a , v , and v_0 , as being related by an equation of the form

$$G(a, v, v_0) = 0, \quad (1)$$

where the function, G , must be such that

$$G(0, u, u) = 0. \quad (2)$$

Under the usual mild restrictions made in deriving theorems on implicit functions, each of the quantities a , v , and v_0 can then be written in terms of the other two:

$$v = b(a, v_0), \quad (3)$$

$$v_0 = h(a, v), \quad (4)$$

$$a = \mu(v, v_0), \quad (5)$$

Clearly, it must be true that

$$b(0, u) = u, \quad (6)$$

i.e., cellular volume at age zero equals the initial volume. Also

$$h(0, y) = y, \quad (7)$$

and

$$\mu(y, y) = 0 \quad (8)$$

(a cell whose volume is the same as the initial volume has age zero). Furthermore,

$$h(x, b(x, u)) = u, \quad (9)$$

that is to say, the initial volume of a cell that has age x and volume $b(x, u)$ is u . Similarly, $h(\mu(y, u), y) = u$ and $b(x, h(x, y)) = b(\mu(y, u), u) = y$.

The equations for the special case of linear volume increase with age are

$$v = b(a, v_0) = v_0 + ra \quad (10)$$

$$v_0 = h(a, v) = v - ra \quad (11)$$

$$a = \mu(v, v_0) = (v - v_0) / r, \quad (12)$$

where r is a positive constant, and for exponential volume increase one has

$$v = b(a, v_0) = v_0 e^{ka}, \quad (13)$$

$$v_0 = h(a, v) = v e^{-ka}, \quad (14)$$

$$a = \mu(v, v_0) = (1/k) \ln(v/v_0), \quad (15)$$

k being another positive constant.

Bell's equation for the age density, $n(t, a, v)$, is as follows:

$$\begin{aligned} \frac{\partial n(t, a, v)}{\partial t} + \frac{\partial n(t, a, v)}{\partial a} + \frac{\partial}{\partial v} [F(a, v) n(t, a, v)] \\ = - [P(a, v) + D(a, v)] n(t, a, v). \end{aligned} \quad (16)$$

This must be supplemented by the equation describing how cells of age zero are added to the population through cell division, namely

$$n(t, 0, v) = 4 \int_a^\infty P(a, 2v) n(t, a, 2v) da. \quad (17)$$

It has been assumed up to this point that all cells with given a and v change their volume at the same rate, $F(a, v)$. Actually, the quantity $F(a, v)$ must be understood as the mean rate of volume increase for such cells. In fact, Fredrickson et al.⁽¹⁴⁾ have shown that although the true rate of change may vary from cell

to cell, it is only the average rate that appears in the final equations. A similar remark was made by Harvey et al. (Reference 7, page 616) after their derivation of the Collins-Richmond equation.

PROGRESS REPORT

Some useful relations between the various derivatives of the functions b , h , and μ can be obtained from Equation (1). According to well-known rules of the calculus

$$\frac{\partial b(a, v_0)}{\partial a} \equiv b_1(a, v_0) = -\frac{G_a}{G_v}, \quad (18)$$

where the subscripts a and v denote differentiation of G with respect to a or v , and the right hand side of Equation (18) must be written as a function of a and v_0 . In the same way,

$$\frac{\partial b(a, v_0)}{\partial v_0} \equiv b_2(a, v_0) = -\frac{G_{v_0}}{G_v}, \quad (19)$$

$$\frac{\partial h(a, v)}{\partial a} \equiv h_1(a, v) = -\frac{G_a}{G_v}, \quad (20)$$

$$\frac{\partial h(a, v)}{\partial v} \equiv h_2(a, v) = -\frac{G_v}{G_{v_0}}, \quad (21)$$

$$\frac{\partial \mu(v, v_0)}{\partial v} \equiv \mu_1(v, v_0) = -\frac{G_v}{G_a}, \quad (22)$$

$$\frac{\partial \mu(v, v_0)}{\partial v_0} \equiv \mu_2(v, v_0) = -\frac{G_{v_0}}{G_a}. \quad (23)$$

Because

$$F(a, v) = b_1(a, h(a, v)), \quad (24)$$

it follows from Equations (18), (20), (21), and (22) that

$$F(a, v) h_2(a, v) + h_1(a, v) = 0 \quad (25)$$

and

$$F(\mu(v, v_0), v) \cdot \mu_1(v, v_0) = 1. \quad (26)$$

Similarly,

$$\mu_2(v, v_0) + \mu_1(v, v_0) b_2(\mu(v, v_0), v_0) = 0 \quad (27)$$

and

$$b_2(a, h(a, v)) \cdot h_2(a, v) = 1. \quad (28)$$

It is convenient to define

$$H(a, v) = \frac{\partial F(a, v)}{\partial v}. \quad (29)$$

Putting

$$b_{12}(a, v_0) = \frac{\partial^2 b(a, v_0)}{\partial a \partial v_0} = \frac{\partial b_1(a, v_0)}{\partial v_0} = -\frac{\partial b_2(a, v_0)}{\partial a}, \quad (30)$$

and using Equation (28) this becomes

$$H(a, v) = b_{12}(a, h(a, v)) \cdot h_2(a, v) = \frac{b_{12}(a, h(a, v))}{b_2(a, h(a, v))}. \quad (31)$$

Consider now the quantity $\int_{x=0}^s H(x, b(x, u)) dx$, where s and u are independent of x . From Equation (31) one has

$$\begin{aligned} \int_{x=0}^s H(x, b(x, u)) dx \\ = \int_{x=0}^s \frac{b_{12}(x, u)}{b_2(x, u)} dx = \ln b_2(s, u) - \ln b_2(0, u). \end{aligned}$$

A little reflection shows that $b_2(0, u) = 1$ (since $v = v_0$ for $a = 0$), and thus

$$\int_0^s H(x, b(x, u)) dx = b_2(s, u). \quad (32)$$

This result, which is the same as Equation (14) of Reference 12, will be used below.

Returning now to Equation (16), it can be written as follows:

$$\begin{aligned} \frac{\partial n(t, a, v)}{\partial t} + \frac{\partial n(t, a, v)}{\partial a} \\ + F(a, v) \frac{\partial n(t, a, v)}{\partial v} = -Z(a, v) n(t, a, v), \end{aligned} \quad (33)$$

with

$$Z(a, v) = P(a, v) + D(a, v) + H(a, v). \quad (34)$$

For a cell population in steady state of exponential growth $n(t, a, v)$ will be of the form

$$n(t, a, v) = e^{ct} \beta(a, v), \quad (35)$$

where c (a positive constant) is the specific growth rate. From Equation (33) one finds for $\beta(a, v)$

$$\begin{aligned} c\beta(a, v) + \frac{\partial \beta(a, v)}{\partial a} \\ + F(a, v) \frac{\partial \beta(a, v)}{\partial v} = -Z(a, v)\beta(a, v). \end{aligned} \quad (36)$$

This equation can be solved by introducing two new independent variables, $\eta (= a)$ and $\zeta (= v_0 = h(a, v))$ instead of a and v . Let $\eta = a$ and $\zeta = h(a, v)$, of which the inverse equations are $a = \eta$, $v = b(\eta, \zeta)$. Also put $\beta(a, v) = \beta(\eta, b(\eta, \zeta)) \equiv \gamma(\eta, \zeta)$, and similarly $Z(a, v) = Z(\eta, b(\eta, \zeta)) \equiv Y(\eta, \zeta)$. Since

$$\begin{aligned} \frac{\partial \beta(a, v)}{\partial a} &= \frac{\partial \gamma(\eta, \zeta)}{\partial \eta} + \frac{\partial \gamma(\eta, \zeta)}{\partial \zeta} \cdot h_1(\eta, b(\eta, \zeta)), \\ F(a, v) \frac{\partial \beta(a, v)}{\partial v} &= \frac{\partial \gamma(\eta, \zeta)}{\partial \zeta} h_2(\eta, b(\eta, \zeta)) \cdot F(\eta, b(\eta, \zeta)), \end{aligned}$$

one obtains from (36), using Equation (25):

$$c\gamma(\eta, \zeta) + \frac{\partial \gamma(\eta, \zeta)}{\partial \eta} + Y(\eta, \zeta) \cdot \gamma(\eta, \zeta) = 0, \quad (37)$$

which gives

$$\gamma(\eta, \zeta) = f(\zeta)e^{-\eta} \exp \left[- \int_{x=0}^{\eta} Y(x, \zeta) dx \right] \quad (38)$$

or

$$\beta(a, v) = K\psi(h(a, v))e^{-\epsilon a} \cdot \exp \left[- \int_{x=0}^a Z(x, b(x, h(a, v))) dx \right], \quad (39)$$

where K is a positive constant and $f(\zeta) \equiv K\psi(\zeta)$ is, so far, an arbitrary function of ζ . However, putting $a = 0$ in Equations (35) and (39) it can be seen that

$$n(t, 0, v) = e^{\epsilon t} \cdot K\psi(v), \quad (40)$$

and therefore $\psi(v)$ must characterize the distribution of birth volumes in the cell population. Hence, it is natural to choose $\psi(v)$ in such a way that

$$\int_{(v)} \psi(v) dv = 1. \quad (41)$$

The constant, K , is then determined from the requirement that the integral of $\beta(a, v)$, Equation (39), over all ages and all volumes must give the initial number of cells in the population, N_0 :

$$\iint_{(a,v)} \beta(a, v) da dv = N_0, \quad (42)$$

and thus

$$\iint_{(a,v)} n(t, a, v) da dv = N_0 e^{\epsilon t}. \quad (43)$$

Combining Equations (17), (35), and (40) it is seen that

$$\psi(v) = \frac{1}{K} \int_{(a)} P(a, 2v) \beta(a, 2v) da. \quad (44)$$

The probability density for the volume distribution of dividing cells, $\varphi(v)$, is related to $\psi(v)$ by the equation

$$\varphi(v) = \frac{1}{2} \psi\left(\frac{v}{2}\right). \quad (45)$$

Hence, it follows from (44) that

$$\varphi(v) = \frac{2}{K} \int_{(a)} P(a, v) \beta(a, v) da, \quad (46)$$

which is intuitively obvious (except for the factor of normalization, $2/K$) from the meaning of P and β .

The expression for $\beta(a, v)$ derived in Equation (39) is valid only if a and v are such that the function $\beta(a, v)$ remains ≥ 0 (or perhaps $\geq \epsilon$, assuming that cells cannot have an initial volume smaller than ϵ). Otherwise $\beta(a, v)$ must be set equal to zero. For example, in the case of linear volume increase [Equations (10)–

(12)] there can be no cell with a given volume, v , and age larger than v/r .

The probability density for the stationary distribution of cellular volumes, i.e., $\lambda(v)$, can now be found by integrating $(1/N_0)\beta(a, v)$ over all ages (the factor $1/N_0$ being introduced to normalize $\lambda(v)$). Thus,

$$\lambda(v) = 2m \int_{(a)} e^{-\epsilon a} \psi(h(a, v)) \cdot e^{-\int_{x=0}^a Z(x, b(x, h(a, v))) dx} da, \quad (47)$$

where m is a constant equal to $K/(2N_0)$. If $u = h(a, v)$ is introduced as a new variable of integration (with constant v) instead of a , Equation (47) becomes

$$\lambda(v) = -2m \int_{u=0}^v e^{-\epsilon \mu(v, u)} \psi(u) B(v, u) \mu_2(v, u) du. \quad (48)$$

Here, $B(v, u)$ is given by the equation

$$B(v, u) = e^{-\int_{x=0}^{\mu(v, u)} Z(x, b(x, u)) dx}. \quad (49)$$

Finally, putting

$$S(v | u) = \exp \left[- \int_{x=0}^{\mu(v, u)} [P(x, b(x, u)) + D(x, b(x, u))] dx \right], \quad (50)$$

one has, with the help of Equations (27), (32), (34), (49), and (50)

$$\begin{aligned} & -B(v, u) \mu_2(v, u) \\ & = -S(v | u) \frac{\mu_2(v, u)}{b_2(\mu(v, u), u)} = S(v | u) \mu_1(v, u). \end{aligned} \quad (51)$$

Therefore,

$$\lambda(v) = 2m \int_{u=0}^v e^{-\epsilon \mu(v, u)} \psi(u) S(v | u) \mu_1(v, u) du. \quad (52)$$

Evidently, $S(v | u)$ is the probability that a cell with volume u at birth will grow to reach volume v without dying or dividing. On this basis, a simple derivation of Equation (47) has been given by A. G. Marr (personal communication) for the special case of negligible cell death (i.e., $D(a, v) = 0$). Then, every cell disappearing from the population is immediately replaced by two new cells, and the birth rate, $\alpha(t)$, must be equal to twice the net increase in number of cells: $\alpha(t) = 2dN(t)/dt = 2\epsilon N_0 e^{\epsilon t}$. A cell present at time, t , with volume, v , and age, a , was born at time, $t - a$, with volume $h(a, v)$. The number of cells found at time, t , with volume between v and $v + dv$, which is $N_0 e^{\epsilon t} \cdot \lambda(v) dv$, must also be obtained by taking the birth rate at time $t - a$, multiplying it by $\psi(h(a, v)) \cdot h_2(a, v) dv \cdot S(v | h(a, v)) [h_2(a, v) dv$ being the volume element that corresponds to dv for cells of age zero] and integrating over all ages. Thus

$$N_0 e^{ct} \lambda(v) dv = \int_{(a)} 2c N_0 e^{c(a-v)} \psi(h(a,v)) h_2(a,v) dv S(v | h(a,v)) da$$

or

$$\lambda(v) = 2c \int_{(a)} e^{-ca} \psi(h(a,v)) \cdot h_2(a,v) S(v | h(a,v)) da. \quad (53)$$

It is readily seen that

$$h_2(a,v) = [b_2(a, h(a,v))]^{-1} = \exp \left[- \int_{x=0}^a H(x, b(x, h(a,v))) dx \right],$$

and it will be shown below that m must be equal to c if $D(a,v) = 0$; hence Equation (53) is the same as Equation (47) for the case of no cell death.

The equation of Collins and Richmond will be obtained using a method suggested by Bell (private communication). First, two functions, $f(v)$ and $d(v)$, are defined by the equations

$$f(v)\lambda(v) = \frac{1}{N_0} \int_{(a)} F(a,v) \beta(a,v) da, \quad (54)$$

$$d(v)\lambda(v) = \frac{1}{N_0} \int_{(a)} D(a,v) \beta(a,v) da. \quad (55)$$

Proceeding as in the derivation of Equation (52), one finds* from Equation (46), (54), and (55):

$$f(v)\lambda(v) = 2m \int_{u=0}^v \psi(u) Q(v,u) du, \quad (56)$$

$$d(v)\lambda(v) = 2m \int_{u=0}^v \psi(u) Q(v,u) D(\mu(v,u), v) \cdot \mu_1(v,u) du, \quad (57)$$

$$\varphi(v) = 2 \int_{u=0}^v \psi(u) Q(v,u) P(\mu(v,u), v) \cdot \mu_1(v,u) du, \quad (58)$$

where

$$Q(v,u) = e^{-c\mu(v,u)} S(v | u), \quad (59)$$

and Equation (26) has been used to simplify the integrand in Equation (56).

Consider now the expression $-\int_{y=0}^v [c\lambda(y) + d(y)\lambda(y) + m\varphi(y)] dy$ which is equal to

* Similarly, Equation (44) is transformed into $\psi(v) = 4 \int_{u=0}^{2v} \psi(u) Q(2v,u) P(\mu(2v,u), 2v) \mu_1(2v,u) du = 2\varphi(2v)$. This is Bell's integral equation [Equation (26) of Reference 12] which must be satisfied by the function $\psi(v)$ in order that a steady state of exponential growth may be attained.

$$-2m \int_{y=0}^v \int_{u=0}^y \psi(u) [c + D(\mu(y,u), y) + P(\mu(y,u), y)] \mu_1(y,u) Q(y,u) du dy. \quad (60)$$

Remembering that

$$\frac{\partial Q(y,u)}{\partial y} = -[c + D(\mu(y,u), y) + P(\mu(y,u), y)] \mu_1(y,u) Q(y,u),$$

and interchanging the order of the two integrations, (60) becomes

$$2m \int_{u=0}^v \psi(u) \int_{y=u}^v \frac{\partial Q(y,u)}{\partial y} dy du$$

or

$$2m \int_{u=0}^v \psi(u) [Q(v,u) - 1] du,$$

since $Q(u,u) = 1$. It has been shown, therefore, that

$$f(v)\lambda(v) = m \int_{y=0}^v [2\psi(y) - \varphi(y)] dy - \int_{y=0}^v [c + d(y)] \lambda(y) dy. \quad (61)$$

Letting v tend to infinity in Equation (61), and assuming $\lim_{v \rightarrow \infty} [f(v)\lambda(v)] = 0$, it can be seen that

$$m = c + \int_{y=0}^{\infty} d(y)\lambda(y) dy, \quad (62)$$

because λ , φ , and ψ are normalized to unity. In particular, if there is no cell death ($D(a,v) = d(v) = 0$), m is equal to c and

$$f(v)\lambda(v) = c \int_{y=0}^v [2\psi(y) - \varphi(y) - \lambda(y)] dy. \quad (63)$$

This is the Collins-Richmond equation which was originally derived in another manner.^(4, 7, 10)

Differentiating both sides of Equation (61) with respect to v one finds

$$\frac{dg(v)}{dv} = m[2\psi(v) - \varphi(v)] - \frac{[c + d(v)]}{f(v)} g(v), \quad (64)$$

where $g(v) = f(v)\lambda(v)$. This equation can be integrated to give $g(v)$, taking $g(0) = 0$ as initial condition. The result is

$$\lambda(v) = \frac{m e^{-R(v)}}{f(v)} \int_{x=0}^v [2\psi(x) - \varphi(x)] e^{R(x)} dx, \quad (65)$$

with

$$R(v) = \int \frac{[c + d(v)]}{f(v)} dv. \quad (66)$$

The right hand sides of Equations (52) and (65) are two different expressions for the same function, $\lambda(v)$. It should be pointed out that Equation (64) can be obtained directly by integrating both sides of Equation (36) over a (cf. Bell and Anderson, Equation (8) of Reference 11).

CONCLUSION

1. For the special case of linear volume increase with age [Equations (10)–(12)], and assuming that $D(a, v) = d(v) = 0$, so that $m = c$, one obtains from Equation (52)

$$\lambda(v) = 2be^{-bv} \int_{u=0}^v \psi(u) S(v|u) e^{bu} du, \quad (67)$$

where

$$b = \frac{c}{r}.$$

Notice that b is also equal to $[\int_{v=0}^{\infty} v \lambda(v) dv]^{-1}$. To see this, multiply both sides of Equation (64) by v , and integrate over v from $v = 0$ to $v = \infty$. The left side is integrated by parts, assuming that $v f(v) \lambda(v)$ tends to zero for $v \rightarrow \infty$. Furthermore, it is clear that $\int_{v=0}^{\infty} v [2\psi(v) - \varphi(v)] dv = 0$, from Equation (45), and so one is left with

$$c \int_{v=0}^{\infty} v \lambda(v) dv = \int_{v=0}^{\infty} f(v) \lambda(v) dv - \int_{v=0}^{\infty} v d(v) \lambda(v) dv \quad (69)$$

[cf. Equation (11) of Reference 11]. If $d(v) = 0$ and $f(v) = r$ this gives $\int_{v=0}^{\infty} v \lambda(v) dv = b^{-1}$.

The mean volume at birth, z , can be defined by the equation* $z = rT = (1/b) \ln 2$, where $T = (1/c) \ln 2$ is the doubling time of the population (so that z is also the volume increment in time T). Thus, Equation (67) becomes, replacing $\psi(u)$ by $2\varphi(2u)$:

$$\lambda(v) = 4b \cdot 2^{-v/z} \int_{u=0}^v \varphi(2u) S(v|u) 2^{u/z} du. \quad (70)$$

If $S(v|u)$ were independent of u (which in general is not true), it could be written in the form

$$1 - \int_{x=0}^v \phi_b(x) dx,$$

and Equation 70 would agree with Kubitschek's result (A-7) of Reference 2 if $\int_{u=0}^v \varphi(2u) 2^{u/z} du$ were equal to $C \int_{x=0}^v \phi_b(2u) 2^{-u/z} du$ (where C is a constant) for all

* This is only approximately true. For example, if there is no cell death and if the function $P(a, v)$ does not depend on v , the correct expression for z is Equation (50) of Reference 15 (in which $\langle v_b(\infty) \rangle$ is the same as z).

values of v ; hence $\varphi(x) 2^{x/z} = \phi_b(x)$. This, however, seems to differ from Equation (25) of Reference 4 (in which the notation $\phi_m(x)$ is used for $\varphi(x)$).

On the other hand, still assuming linear volume increase with age and no cell death, Equation (65) gives

$$\lambda(v) = be^{-bv} \int_{x=0}^v [4\varphi(2x) - \varphi(x)] e^{bx} dx. \quad (71)$$

2. Consider now the case of exponential volume increase with age [Equations (13)–(15)] and assume $D(a, v) = d(v) = 0$. It can readily be seen that k must be equal to c . This conclusion is reached by putting $d(v) = 0$ and $f(v) = kv$ in Equation (69). Thus, one has from Equation (52)

$$\lambda(v) = \frac{4}{v^2} \int_{u=0}^v u \varphi(2u) S(v|u) du, \quad (72)$$

which again differs from Kubitschek's result (A-8) of Reference 2, even if $S(v|u)$ is replaced by

$$1 - \int_{x=0}^v \phi_b(x) dx.$$

Using instead Equation (65), one finds

$$\lambda(v) = \frac{1}{v^2} \int_{x=0}^v [4\varphi(2x) - \varphi(x)] x dx \quad (73)$$

for the case of exponential volume increase with age and no cell death.

In general, the expressions obtained by the method of Collins and Richmond [Equations (71) and (73) above] are easier to use in practice, particularly if cell death is negligible. This is because $S(v|u)$ is an unknown function, for all practical purposes, whereas $\varphi(v)$ can be determined experimentally - a fact that has been brilliantly exploited by Anderson et al.⁽¹⁹⁾

It would be interesting to try to fit Kubitschek's results⁽²⁾ using Equations (71) and (73) in place of his (A-7) and (A-8). Preferably, $\varphi(v)$ should be found by means of suitable experiments; otherwise it would be necessary to make a specific assumption on the shape of $\varphi(v)$. For example, it might be supposed that reciprocal volumes at division are normally distributed, which is analogous to what Kubitschek did for his function $\phi_b(x)$.

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CHARACTERIZATION OF PHOTOREACTIVE PROCESSES IN BACTERIA

Sylvanus A. Tyler and Merlin H. Dipert

PURPOSE AND METHODS

A model of the kinetics of photoreactivation in UV-irradiated bacteria was presented in the previous annual report.⁽¹⁾ The underlying postulate of this model states that UV-irradiation of the cell produces events (among other lesions) within the cell with the following properties: 1) A single event can inactivate the cell; 2) events are sequentially reversed* by exposure to visible light; 3) the cell is reactivated if, and only if, all events sustained are reversed. The events are called *reversible events* to distinguish them from other induced lesions that also may influence and contribute to the *total surviving fraction* of the population. The proportion (Q_r) of reversible events that contributes to the total proportion (Q_T) of inactivated cells must be independent of all other inactivating events (proportion = Q_i). This condition establishes the relationship

$$P_T = P_r \cdot P_i \text{ for } P = 1 - Q.$$

The description, based on the above postulation, was called "a sequential model."[†] In addition, it was assumed that the events produced by a dose d of UV irradiation are Poisson-distributed; i.e.,

* By reversal we mean any counter-action that nullifies, repairs, or circumvents the effect of an induced lesion that measurably contributes to the end point considered.

† Dr. David J. G. Davies, School of Pharmacy, Bath University of Technology, Bath, England originally proposed the sequential model of photoreactivation and supplied the experimental data upon which this report is based.

$$\text{Prob. } [x; \mu(d)] = \frac{[\mu(d)]^x e^{-\mu(d)}}{x!}, \quad (1)$$

where x and $\mu(d)$ are the number and expected value of events produced, respectively. We refer to this model, together with the assumption of Poisson-distributed events, as the *Poisson-Sequential Model*. The observed total surviving fraction, $[S(d,t)]$, after an exposure for time, t , to visible light follows a dose, d , of UV irradiation is*

$$S(d,t) = P_T(d) \left\{ \sum_{i=0}^k \frac{[\mu(d)]^i}{i!} + \frac{\gamma[\mu(d)]^{k+1}}{(k+1)!} \right\}, \quad (2)$$

where $P_T(d)$ is the surviving proportion after zero photoreactivation, $\mu(d) = -\ln[P_r(d)]$, γ is a positive constant (less than 1), and $L = k + \gamma$ is the number of reactivated events. This model is compatible with the survival data⁽²⁾ accumulated over a range of t and d values for cells of *E. coli* WP2 her⁻ in stationary phase at photoreactivating irradiances of 60, 4000, and 6000 ergs mm⁻² sec⁻¹. It was found that at each irradiance not more than approximately 20 events could be reversed. This suggests that only a prescribed number (say, 20) of reversible events are produced by UV irradiation and that a Binomial-Sequential Model may serve as a more appropriate description of the kinetics of photoreactivation.

* In Reference 1, $P_i(d)$ should be changed to $P_T(d)$ in Equations (5) and (6).

PROGRESS REPORT

The Binomial-Sequential Model

Let us substitute for the Poisson assumption [Equation (1)] that, at most, n reversible events are produced by the irradiation and are binomially distributed within the cell with probability $(1 - \rho)$. Then,

$$\text{Prob. } [x:n, 1 - \rho] = \binom{n}{x} (1 - \rho)^x \rho^{n-x}, \quad (3)$$

where x is the number of events produced, and

$$\binom{n}{x} = \frac{n!}{x!(n-x)!}.$$

The observed total surviving fraction is expressed by

$$S(d, t) = P_i(d) \left\{ \sum_{x=0}^k \binom{n}{x} (1 - \rho)^x \rho^{n-x} + \gamma \binom{n}{k+1} (1 - \rho)^{k+1} \rho^{n-k-1} \right\}, \quad (4)$$

where ρ is equal to the n th root of P_r and k is the largest integer for which the first term on the right of (4) does not exceed $S(d, t)$. With $n = 20$, the Binomial-Sequential Model was indistinguishable statistically from the Poisson-Sequential Model when the same data from the experiment with *E. coli* WP2 her⁻ were used.⁽²⁾ However, this model requires the a priori determination of n for processing. Thus, an iterative numerical procedure based on some criterion of goodness of fit must be employed to estimate n . Several criteria are under study currently.

Random Models

The efficiency of two random models as descriptions of the kinetics of photoreactivation was tested. The structure of these models rests on the postulation that events are *randomly* reactivated rather than *sequentially* reactivated by light. The Poisson-Random model depicts the change in total surviving fraction with UV dose and photoreactivation time by the following expression:

$$S(d, t) = P_i(d) \sum_{x=0}^{\infty} \frac{[\mu(d)]^x e^{-\mu(d)} P_h^x}{x!} \quad (5)$$

$$= P_i(d) \{ e^{-\mu(d)[1-P_h]} \},$$

where P_h is the probability of reactivating an event and $\mu(d) = -\ln[P_r(d)]$. The Binomial-Random Model is symbolically described by

$$S(d, t) = P_i(d) \sum_{x=0}^n \binom{n}{x} (1 - \rho)^x \rho^{n-x} P_h^x \quad (6)$$

$$= P_i(d) \{ (1 - \rho)P_h + \rho \}^n,$$

where ρ is the n th root of P_r .

FIG. 58.—Flow chart for the construction of survival curves

With several different strains of *E. coli*, the Binomial-Sequential Model seems to provide the best description of the observed surviving fractions. We have noticed, however, that if a random model describes the data well, the Binomial-Sequential model will fit even better provided n is small (i.e., $n \approx 5$). Future efforts will be directed toward a more systematic investigation of these impressions.

The elucidation of a model for which empirical validation is sought is not practically described until the computational algorithms that make the necessary statistical testing feasible are established. Thus, along with the construction of these models, the numerical analysis and coding of supporting computer programs have proceeded.

The computer algorithms for determining the constants of the model are summarized in the flow chart (Figure 58). Although the detailed procedures represented by each box will change as the particulars of the model are further developed, the essential elements will remain.

The steps are:

1. Compute the base line parameters for no photoreactivation and maximum photoreactivation. Compute the surviving averages.
2. Compute the number of events reversed (L) and the probabilities (P_h) using either Equations (2), (4), (5), and (6).
3. Find the mean L and P_h for plotting.
4. Plot the theoretical survival curves and the observed points.

The present algorithms will be extended to permit the statistical estimation of the characterizing con-

stants of the models by minimum variance, least square procedures.

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A THREE-DIMENSIONAL MODEL FOR RHYTHMIC FLOWERING CYCLES IN XANTHIUM

Merlin H. Dipert, William Chorney, and Richard R. Dedolph

PURPOSE AND METHODS

The development of a mathematical model to obtain a quantitative description of the flowering cycle of *Xanthium* was outlined in the last report.⁽¹⁾

PROGRESS REPORT

New data were obtained from carefully replicated experiments with light interruption times at 0, 4, 8, 12, 16, 20, and 24 hr. Analysis and plotting, using the two-dimensional model [Equation (1), Reference 1] showed the 0- and 4-hr interruption data were significantly different from the 8- to 24-hr data, and they were not used in the three dimensional analyses.

Three-dimensional analysis [Equation (2), Reference 1] of the 8- to 24-hr data showed that parameters P_7 , P_8 , P_9 and P_{11} were not significantly different from 0. The results of fitting a surface to these data are shown in Figure 59. This surface reduced the sums of squares of the data set by 79%, which well describes the data trends of flowering with respect to the x (time) and z (light interruption treatments) axes.

The response surface is a damped sine wave with a small negative slope in the x direction, with ridges and troughs when both rise in the xz direction and are uniformly displaced as a function of z .

To establish the location and direction of these

FIG. 59. A three-dimensional model for rhythmic flowering cycles in *Xanthium*.

TABLE 30. COEFFICIENTS AND STANDARD ERRORS ASSOCIATED WITH A DAMPED HARMONIC FUNCTION DESCRIBING FLOWERING IN *Xanthium*

	Parameter	Standard error
P_1	4.57	0.34
P_2	-0.069	0.009
P_3	7.85	1.77
P_4	-0.038	0.007
P_5	-4.01	0.20
P_6	0.22	0.006
P_{10}	-0.19	0.014
P_{12}	0.003	0.0003
Period	29.1	0.80

ridges (maxima) and troughs (minima), the x and z coordinates were calculated from an approximation formula.

Given any value z ,

$$x = \left[n\pi + \tan^{-1} \left(\frac{-P_6}{P_4} \right) - P_5 - P_{10}z \right] / P_6,$$

$$n = -1, 0, 1, 2, 3$$

The parameters of the surface and their standard errors are shown in Table 30. These values are in full agreement with the corresponding values calculated for the two-dimensional model of these data.

CONCLUSION

We have described techniques making possible the valid interpretation of the combined effect of light interruption and darkness duration on the flowering of *Xanthium*. This response surface forms the basis for critical interpretation of the physiology of these treatments in inducing this long-studied phenomenon. These techniques have worked well in describing this complex response and have great utility investigating other rhythmic data of either a two- or the three-dimensional nature.

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JANUS: STATUS OF THE FACILITY AND TECHNICAL CONSIDERATIONS

Frank S. Williamson

PURPOSE AND METHOD

Previous reports have traced the history of the JANUS irradiation facility, the operational problems encountered, and the eventual shutdown for modification on October 18, 1968.^(1,2) Experimental evaluations of the proposed shielding changes to the shutters and the walls, floor and ceiling of the High Flux Room have been described.^(3,4)

These modifications include a) constructing new shutters and shutter-pedestals of optimum shielding design and maximum allowable thickness, b) fabricating a new U^{235} converter plate and installing it on the High Flux Room side of the shutter location, primarily to release space for a thicker shutter, and c) lining the High Flux Room itself with a layer of lead 4 in thick, backed by 4 in of a neutron absorbing material.

PROGRESS REPORT

The JANUS High Flux Room

Detailed designs for the modifications have been prepared by personnel of Reactor Operations and Plant Engineering Divisions, under the guidance of a committee consisting of individuals from these, and the Biology and the Reactor Physics Divisions. Design improvements have been introduced since the modifications began. The original plan⁽¹⁾ involved a 4-in layer of borobauxite concrete behind the lead room liner. Accelerated curing tests were carried out on samples of this material in a vacuum chamber in order to determine the residual water content. The final value of approximately 2.9×10^{22} atoms of H/cc was about $1/3$ of that originally sought for hydrogen concentration (8×10^{22} atoms of H/cc).

At this time, discussions with the Masonite Corporation indicated that a boron-impregnated hardboard could be produced with the desired characteristics, and samples of hardboard stock were tested for neutron activation. The best results were obtained from a hardboard made from redwood fibers, and the final product, Benelex 207, consisted of such a board impregnated with ammonium diborate. This material has 2.5% boron by weight with only 125

parts per million of sodium, and 6% by weight, or 3.6×10^{22} atoms/cc, of hydrogen.

Tests of activation by thermal neutrons indicated a factor of improvement over borobauxite concrete of approximately 30. In addition to some superiority over the borobauxite concrete as regards hydrogen concentrations and neutron activation, the Benelex 207, in the form of planks 1 in thick and 16 in wide, is much easier to erect as a wall liner.

A design difficulty which had been appreciated from the inception of the modification proposal was that of supporting the 4-in lead ceiling without presenting neutron-activatable material on the inside of the High Flux Room. This problem was solved by making the ceiling of 12-in square "tiles" of 4-in thickness, each weighing 240 pounds and having four $1/4$ -in diameter aluminum studs cast in to act as hangers. These tiles are suspended from an aluminum gridwork, which in turn is supported by the bottom flanges of steel I-beams. The steel was specified for low manganese and cobalt content. The space above the lead tiles is filled with borobauxite concrete which, while questionable in a 4-in layer, is adequate in the 8-in thickness.⁽⁵⁾

This concrete, which is loaded with boron carbide, minimizes the thermal-neutron activation of steel and aluminum support structures, except for the bottom flanges of the steel I-beams, which are immediately adjacent to the lead ceiling. In order to correct this situation these flanges were coated with a paint, made in the laboratory, consisting of gadolinium oxide in a vehicle of polyurethane varnish.

Delays have occurred due to difficulties experienced by the contractor in installing the lead ceilings, and by the Central Shops Department in machining the boron-loaded polyethylene blocks used in the shutters and shutter pedestals. At the date of this report, October 1969, the lead walls and ceiling are completed. The floor will be left until heavy traffic in the room has ceased. A revised schedule projects startup of the reactor for low-power testing in early December, at which time the neutron flux distribution across the converter plate will be measured so that a graded attenuator can be designed. It is important not to produce inconvenient activation of the converter plate

before this attenuator is installed, tested, and if necessary, corrected. Initially, therefore, all spectrometry, dosimetry studies and other testing which require the use of the converter plate must be carried out at low power and can begin early in 1970.

It is intended to house mice, during irradiation, in disposable polyethylene containers loaded into removable shelves in a modular frame which will then be hung in the High Flux Room and identified by the data-acquisition system. Calculations by Shaftman and McArthy⁽⁵⁾ indicate that the thermal neutron flux in the modified High Flux Room during irradiation will be greatly reduced. If this flux is taken as unity, then the presence of 500 mice in polyethylene containers will raise the thermal neutron flux by a factor of 28, and 1000 mice by a factor of 56. The 500 mice are expected to produce approximately the same thermal neutron flux as there was in the old, unmodified room without an animal loading.

In order to further minimize hazards to persons handling these irradiated frames the materials have been chosen so as to combine minimum activation with rapid decay. A prototype is under construction, using magnesium-aluminum alloy. It is not expected that activation due to neutron capture will present a problem, but, should this aspect require attention, the offending material can be treated with gadolinium paint. Although the polyethylene vehicle forms a tough, pliable film, an epoxy resin vehicle might be even better.

Automation and Data Processing

Because of a series of budgetary difficulties, it has not yet been possible to obtain the data acquisition system necessary for a large throughput of mice.

Experiments have been carried out on file management, using data collected in another research program. The most promising file organization is Indexed Sequential, supported by the IBM 360 50-75 operating system in the ANL Computing Center. In this type of file, the records are arranged by a collating sequence of identification keys and indexed. Additions and retrievals may be made at random with reasonable efficiency, but sequential search of the entire file is very efficient. With Release 17 of the operating system, it became possible to have access an entire group of records by specifying that part of the identification key common to the group. These features ensure the flexibility necessary if the data processing goal is to be achieved. This goal is to create only general programs which can be controlled or modified at execution time in order to achieve the desired result.

Use of expert programming staff to write special

programs whenever data must be updated or retrieved and routine statistics and plotting operations are needed is not acceptable. These functions should be carried out by the scientist, using simple commands approximating normal language.

The PL 1 language is our standard higher language for all JANUS programming, with some assistance from subroutines written in OS/360 Assembler language, where this is justified by an improvement in execution efficiency or the objective is otherwise unattainable.

A teletype remote terminal, linked by telephone to the RESCUE interactive system on the 360 50, is now in use, and an IBM 2741 terminal is on order. The 2741 resembles a Selectric typewriter and will be appropriate for use by a secretary to enter data into the computer directly.

JANUS data files will be maintained on disk or data cell storage by the 360 75 and updated through the RESCUE system and a batch computing job. Retrievals, if brief, can be read back at the 2741 terminal, or if more voluminous, directed to the local printer (scheduled for installation in building 202 in spring 1970) or main printers in the Computing Center.

A flexible automatic graph plotting system is under development. Eventually, it will be possible to produce plots on paper or film whenever data are retrieved from the files, or computed, without any special attention. A first version, restricted to Calcomp plotting, is now operating from commands and data entered into RESCUE by teletype.

CONCLUSION

The JANUS reactor is expected to be in operation early in December 1969. Evaluations of the flux distribution will begin immediately thereafter. Spectrometry and dosimetry will be carried out in parallel with the initial radiobiology studies early in 1970.

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THE JANUS RADIOBIOLOGY PROGRAM

E. John Ainsworth

The JANUS program is an integrated experimental approach to the evaluation of hazards associated with short- and long-term exposure to fission spectrum neutrons or gamma radiations. The objective is to define radiation responses that include both acute lethality and late effects, such as neoplasia and degenerative diseases, in terms of radiation damage to cells, alterations in the functional integrity of tissues and organ systems, and decrements in physiological capacity. In this way, it will be possible to predict more meaningfully and compare the consequences of human exposure, occupational or otherwise, to fission spectrum neutrons or gamma radiation. The present experimental planning for the first year concerns mice and, to a limited extent, dogs. The main thrust of the program focuses on late effects of irradiation, but acute lethality studies must be performed to provide essential baseline information. Experiments are planned to evaluate the extent to which repair and recovery phenomena influence both late effects and the short-term survival hazard attendant to repeated low level exposures.

The initial range-finding experiment in the late effects sector of this program seeks essential baseline data concerning life-span shortening produced by a single acute exposure to gamma or neutron radiation; graded exposures will be used to provide dose-response relationships. A directly comparable fractionation experiment is planned in which a high total exposure, viz., 300 Rads of neutrons and 900 Rads of gamma radiation, is protracted from several days to approximately 180 days. From these experiments, certain late effects of radiation, viz., life-span shortening, death rate, tumor incidence and type can be evaluated as a function of total dose, the extent of dose fractionation, and radiation-free time. Morbidity groups must be included so that, by serial sacrifice, information can be obtained about the development of radiation lesions and functional impairments, the ultimate mechanisms that produce deleterious late effects of irradiation.

A small initial effort is directed to acute lethality studies in several mouse strains to characterize the relative contributions of hematopoietic and intestinal damage to the neutron radiation syndrome and to explore dose-rate (exposure-time) effects. Comparable studies are planned for dogs. Improved techniques to evaluate damage to stem cells in the mouse marrow

and intestinal crypts^(1,2) and a thorough examination of cell population kinetics in the intestine⁽³⁾ should provide a better definition of the neutron syndrome essential to the interpretation of results from other studies of fractionated or chronic exposures.

Recovery in mice after single or multiple acute exposures will be measured by split-dose determination of the LD_{50/30} and LD_{50/5}. The accumulation of injury during fractionated exposure will be evaluated by determination of multiple exposure LD₅₀'s and by the split dose LD₅₀ method. Hematological experiments in support of multiple exposure studies will contribute better understanding of the continuum of syndromes occurring under conditions of long-term exposure, and the extent to which the multiple exposure LD₅₀ can be predicted from functional indices of radiation injury to the hematopoietic system.

The current status of the modifications of the High Level Exposure Room are described in an accompanying report.⁽⁴⁾ Although the time required to assemble and test the various reactor components and obtain full sanction for unlimited operation of the facility cannot be predicted at this writing, critical dosimetric experiments will be conducted coincidental to tests of the facility. The more extensive dosimetry is to be coordinated with the initial radiobiology effort to determine the influence of spectral variations on survival of Chinese hamster cells exposed *in vitro*, weight loss in mouse testes, and survival of mice and their hematopoietic stem cells. These biological dosimetry experiments, to be completed within a few months of reactor operation, are designed to define exposure constraints within the High Level Room imposed by energy dependence of biological response. Initiation of all other experiments described above is planned for the first year.

Earlier ANL toxicity programs, concerned primarily with duration-of-life exposures, have generated basic radiobiological principles, testable hypotheses, and extrapolations pertinent to the hazards of human exposure.⁽⁵⁻⁷⁾ The JANUS radiobiology program, which is equally ambitious in its objectives, is the recipient of this earlier experience and a perspective which recognizes that only by an integrated approach, involving both mortality and comprehensive studies of cellular and tissue injury, can the hazards of neutron and gamma irradiation be evaluated and meaningfully predicted.

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Studies of Radiation-Induced Changes

TOXICITY AND METABOLISM OF RADIONUCLIDES: PROGRAM OBJECTIVES AND INTRODUCTION

William P. Norris

For a long time it has been recognized that reactor accidents and nuclear detonations are potential sources of radiation exposure to large segments of the population. A dosimetric reconstruction of such an exposure would obviously be very complex, since there will be contributions from many sources, including inhaled and ingested fission products. In spite of the concern expressed over our lack of information in this area, there have been no direct experimental attacks on the problem. While there are data to describe radiation damage resulting from experimental exposures to single radionuclides, these are not directly, or necessarily, applicable to fission mixtures.

Some persons have supported the straight-away approach to the fission mixture problem—that is, a study of animals treated with raw mixtures of fission products. However, we rejected this suggestion on the grounds that such a complex mixture is impossible to deal with either analytically, or conceptually. Furthermore, it is impractical to attempt to reproduce the composition of a given fission mixture, because it depends so heavily on the fuel source, time in the reactor, and postirradiation cooling time. Therefore, we devised an alternative, technically feasible approach, which should contribute to the definition of the biological problem associated with fission mixtures.

The fission mixture problem can be restated to ask what will happen if different portions of the body are unequally, but more or less simultaneously, irradiated. Because radioactive isotopes are involved, both the total doses and the dose rates to areas of the body may vary. A practical example exists in the natives of Rongelap, who received whole-body irradiation from ground deposits of fission products together with significant doses to the thyroid gland from metabolized ^{131}I .

The radiation dose delivered to the body from a radioactive isotope deposited internally is intimately related to the metabolic characteristics of the isotope

and the quality of its emitted radiations. The total dose to the body is protracted, but the dose rate decreases with time, depending upon the biologic rate of excretion and the physical rate of decay of the isotope in question.

Some elements, such as cesium, maintain an essentially constant relative distribution between the organs of the body; others, such as cerium, may show high initial concentrations in one organ with subsequent translocation to another.

It is possible to select a few fission products, possessing markedly different metabolic characteristics, which typify the biochemical properties of the majority of radionuclides in a fission mixture. For our immediate purposes these are ^{137}Cs , ^{144}Ce , ^{90}Sr , and ^{131}I . These radionuclides must first be studied separately to determine their acute lethality, and the biological response to doses below those which are acutely lethal. Such data for ^{90}Sr will be obtained from work of other laboratories.

In addition to the above group of internal emitters, whole- or partial-body irradiation from calibrated external sources will be used to provide base-line information on the response of dogs under known dosimetry. Such exposures will include whole-body irradiation to determine the single-dose $\text{LD}_{50/30}$, and protracted irradiation at both constant and decrementing dose rates. Such studies should do much to relate the response of the dog to the larger volume of work already done in rodents. A comparison of these data should determine whether a simple ratio of radiosensitivity can be used to extrapolate between species under various exposure conditions.

As implied above, the next step would be to investigate the effects of simple combinations of these radiation exposure—for example, protracted whole-body irradiation combined with ^{131}I administration. The biologic rationale underlying such exposures appears to be evident.

An adequate understanding of radiation effects

from a given fission product must include a detailed knowledge of the dynamics of its metabolism, together with the capability to convert this information into meaningful estimates of radiation dose rate and integrated radiation dose to significant organs and tissues. The latter is a difficult problem, in which limited success has been reported to date. However, the application of a combination of available dosimetric techniques—some of which have already been explored here—should contribute significantly in this most important area. Unless the importance of dosimetry is recognized, the problems associated with internally deposited isotopes will never be adequately resolved.

One great advantage of the dog lies in its size,

which allows it to be examined repeatedly and in detail. However, interpretation of results requires a comprehensive knowledge of the normal, unirradiated animal, and also of the changes which occur as the animal matures and ages. Thus, a study of all aspects of the normal dog is equally as important as a study of the irradiated animal.

The collection of such information—if it is to be meaningful after many years—must be highly systematized to allow for continuity of observations entered in a comparable manner. The only feasible method of retrieval is through a computer. Thus, computer compatible data entry systems were regarded as necessary from the beginning.

THE RESPONSE OF ANL BEAGLES TO PROTRACTED EXPOSURE TO ^{60}Co γ RAYS AT 5 TO 35 R/DAY. I. SURVIVAL AND CLINICAL OBSERVATIONS

William P. Norris and Calvin M. Pool

PURPOSE AND METHODS

Our Annual Report for 1968⁽¹⁾ described the initiation of an experiment in which young adult beagle dogs are maintained in a ^{60}Co γ -ray field until death. The daily exposure rates are either 35, 17, 10, or 5 R/22-hr exposure day. These exposures are estimated to be equivalent to either 25.40, 12.34, 7.26, or 3.63 Rads/day, respectively.

The capacity of the ^{60}Co γ -ray facility, as arranged for this experiment, is 52 dogs—4 at 35 R/day, 8 at

17 R/day, 16 at 10 R/day, and 24 at 5 R/day. As the dogs died, they were replaced by others, so the total number of dogs irradiated to date is in excess of 52.

Each dog is examined regularly for clinical abnormalities, with special attention to the eyes (for signs of retinal pathology and lens opacities) and body temperature (for indications of septicemia).⁽²⁾ A complete hematologic work-up, including a differential white cell count, enumeration of platelets and reticulocytes, and measurement of certain biochemical parameters, is done routinely on each animal. Bacteriologic examinations are performed at intervals on blood specimens from living dogs, as well as on postmortem specimens, to define the significance of bacteremia in the overall response. All decedents are necropsied, and tissues are collected for microscopic study.

PROGRESS REPORT

A summary of survival data obtained to date is presented in Table 31. At 35 R/day, death occurred after an average of 57 days' exposure—a total of 1995 R. At 17 R/day, about $\frac{3}{4}$ of the dogs died after an average of 163 days' exposure (2771 R), but 2 animals are still surviving in apparent good health after 570 days (9690 R) at this exposure rate. Thus, based on survival time, the 17 R/day group has segregated into two subpopulations, one being significantly more radioresistant than the other. A similar pattern appears to be developing in the dogs exposed to 10 R/day. About $\frac{1}{2}$ of these dogs died after an average of

TABLE 31. SURVIVAL OF YOUNG ADULT BEAGLE DOGS MAINTAINED IN A ^{60}Co γ -RAY FIELD

Exposure, R/day	Group	No. of dogs	Results to 9 15 69			
			Decedents		Survivors	
			Survival range, days	Mean	No. dogs	Survival to date, days
35	A & B	8	45-68	57	None	—
17	A	8	85-302	163	2	570
	B	5	131-213		1	353
10	A	8	259-383	304	4	569
	B	8	209-504		3	555
	C	7			7	212
5	A	8	390		7	568
	B & C	16	484-		15	553
Control		8	303-		7	570

FIG. 60.—Mortality vs. total accumulated exposure in roentgens in dogs maintained continuously until death in a ^{60}Co γ -ray field at exposure rates of either 35, 17, 10, or 5 R/day. The arrows indicate the mortality-total exposure relationship at the time this chart was prepared (after 510 days of exposure). The exposures are being continued.

304 days' exposure (3040 R). The rest are still alive after about 560 days.

Exposure of dogs to 5 R/day has not proceeded to the point where mortality data are meaningful.

CONCLUSION

The relationship between mortality and total accumulated exposure at each of these 4 daily exposure rates is presented in Figure 60. We conclude that there are inherent differences in marrow cell regenerative and repair capabilities among individual dogs exposed continuously to irradiation. These capabilities become largely inoperable when the exposure rate is appreciably in excess of 17 R/day.

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THE RESPONSE OF ANL BEAGLES TO PROTRACTED EXPOSURE TO ^{60}Co γ RAYS AT 5 TO 35 R DAY. II. ESTIMATION OF THE LD_{50} AT 35 R DAY

William P. Norris and Calvin M. Poole

PURPOSE AND METHODS

The study of the effects of continuous exposure of animals to ionizing radiations is complicated, because it is usually necessary to regard some terminal portion of the exposure as "wasted"—that is, not contributing to the effect observed. It is useful, therefore, to estimate the LD_{50} in experiments where continuous exposures are terminated at the appropriate time. Such an estimate was attempted for beagle dogs exposed to 35 R/day.

A study of the response of beagles to unterminated exposures to 35 R/day suggested that the LD_{50} is reached after 40 days in such a γ -ray field. Young adult beagles were given such an exposure in groups of 4. A total of 40 dogs has been treated in this manner to date.

PROGRESS REPORT

The mortality and survival data are presented in Table 32. Thirty percent of the dogs failed to survive the 40-day period of continuous exposure to 35 R/day. Of the 28 dogs that did survive the 40-day exposure period (a total of 1400 R), 15 are still surviving. This

TABLE 32. MORTALITY IN BEAGLE DOGS GIVEN PROTRACTED WHOLE-BODY EXPOSURE TO ^{60}Co γ RAYS FOR 40 DAYS

	No. dogs	Survival time ^a	
		Range	Mean
Total Dogs Exposed	40		
1. Failed to survive the 40-day exposure period	12	31-40	37
2. Survived exposure period but died acutely	13	42-63	51
3. Surviving	15 (37.5%)		

^a All times are computed from beginning of exposure. Data tabulated to 9-15-69.

exposure is a close approximation of the LD_{50} without "wasted" radiation under the conditions studied. In almost every instance the prime cause of death was septicemia.

THE RESPONSE OF ANL BEAGLES TO PROTRACTED EXPOSURE TO ^{60}Co γ RAYS AT 5 TO 35 R DAY. III. HEMATOLOGY

Carl E. Rehfeld, Donald E. Doyle, Dorothy L. Chladek,
Donald L. Pearson, and Patrick H. Polk

PURPOSE AND METHODS

The response of the beagle hemopoietic system to continuous ^{60}Co radiation has been recorded for 590 days by systematically enumerating the venous blood cells. The exposure rates are 35, 17, 10, and 5 R/day. Blood samples were obtained at either 7- or 14-day intervals depending upon dose level and rate of change in cellular levels. One new animal classification has been added within the last year: this is the group of dogs recovering from exposure to 35 R day for 40 days. The erythrocyte and leukocyte counts were obtained by electronic counting procedures. Thrombocytes are counted by hemocytometry, and reticulocytes are enumerated in a conventional, vital-staining technique.

The dogs irradiated at the rate of 17 R day have a broad range in survival time and leukopoietic response. Failure to survive constant exposure at this level of irradiation is characterized by gradual depletion of venous cells. The two dogs that have survived nearly 600 days at 17 R/day have cell levels approximately equivalent to the dogs exposed at rates of 10 and 5 R/day.

PROGRESS REPORT

A number of studies have been conducted with mice and rats that include the same dose rates with an exposure time of 23 to 24 hours/day.⁽¹⁻⁴⁾ One of these reports⁽¹⁾ includes the effect of 16 Rads/day (lifetime) on the hemopoietic response in hybrid rats. There was a transitory decrease in mononuclear cells between 15 and 20 days and a lesser effect in the same period for polymorphonuclear cells. In general, cell numbers were little changed at a dose rate of 16 Rads/day over a period of 320 days.

The beagle is more radiosensitive, in some respects, than the rat and has shown a marked hemopoietic response to a dose rate of 17 R/day (Figure 61). The total leukocyte count slowly declined to a 10% level after 115 days of continuous radiation. The number of venous leukocytes increased gradually from a 28% to a 40% level between 220 and 440 days. The curve for 17 R dogs, after about 170 days, is the mean value of only two dogs that have survived the entire period. These two dogs have maintained essentially normal red cell numbers, so the period of decline in the mean

value (Figure 62) was caused primarily by the non-surviving dogs.

Leukocytes in the dogs given 5 R/day gradually decreased in number from a 70% level at 30 days to a 40% level at 340 days, and have maintained at the latter point, or somewhat higher, for about the last 250 days. Dogs submitted to 10 R/day were gradually depleted to a 20% level in about 200 days but have gradually recovered to the 40% to 50% level, starting at 450 days. A number of dogs have recovered after 40 days' exposure at the rate of 35 R/day; 180 days after removal from the radiation field these dogs have an average of 8500 leukocytes/mm³, and the trend is still upward.

There is a direct relation between depression of leukocytes and dose levels until about 115 days; at that time, the 17 R dogs began recovery so that by 190 days the numbers in both 17 R and 10 R dogs began a parallel course. In the period from 350 to 590 days, there have been approximately equivalent numbers of venous leukocytes in the dogs given 17 R, 10 R, and 5 R.

Numbers of leukocytes in recovery or maintenance periods have been described above as percentages of pre-irradiation levels. These percentile figures must be regarded as conservative because of the rather remarkable and persistent low normal values found in the control dogs. In the period from 100 days to 590 days the control values have varied around a level of 8,500/mm³, although they had an average of over 12,000 cells prior to caging. One would expect that the dogs in the irradiation field would also demonstrate this environmental effect. If allowance is made for environmental influence, the dogs given 17 R, 10 R, and 5 R all have 50% to 60% of the normal number of venous leukocytes during the last 200 days.

In Figure 62, as in Figure 61, there are two groups of dogs at 35 R/day; some remained in the radiation field to death, and others were removed after 40 days; comments will be limited to those removed after 40 days. The number of erythrocytes in control dogs was 8.5×10^6 /mm³ before caging and have remained at 8.0×10^6 /mm³ since 100 days. Erythrocyte numbers were essentially unchanged in dogs exposed at 5 R/day. There was a long, gradual decline and recovery period between 100 and 400 days in dogs exposed at 10 R/day; the low point of 5.3×10^6 /mm³ was ob-

FIG. 61. Effect of ^{60}Co gamma rays upon the number of venous leukocytes in beagles at exposure rates of 5 R, 10 R, 17 R, and 35 R/day. One group was exposed to 35 R/day until death and the other terminated after 40 days.

FIG. 62. Effect of ^{60}Co gamma rays upon the number of venous erythrocytes in beagles at exposure rates of 5 R, 10 R, 17 R, and 35 R/day. One group was exposed to 35 R/day until death and the other terminated after 40 days.

served at about 250 days. The 17 R dogs reached a low point of 4 million cells at 170 days in a decline and recovery period from 100 days to 370 days. The 35 R dogs made a rapid recovery after their removal from the irradiation field and have maintained the normal range from 110 to 180 days since their removal.

Transient macrocytosis (erythrocytes) was observed in three groups of dogs (Figure 63). In the 35 R dogs, where exposure was terminated at 40 days, there was a significant increase in mean cell volume (MCV) in the period 20 to 120 days after the end of exposure. The 17 R dogs had an increased MCV from 140 to 300 days, and the 10 R dogs were affected between 220 and 340 days. Increased MCV and reticulocyte counts were not necessarily related (Figure 63).

Reticulocyte numbers varied considerably within short periods of time (Figure 63); however, the 10 R

and 5 R dogs were quite similar in response. The 17 R dogs had a 100-day period at the 0.02% level starting about 85 days followed by a 1.00% level between 200 and 300 days, with a repetition of this cycle during the past year. During the first 300 days the control values were generally above or equal to those of irradiated dogs, but during the last 300 days the controls have been below or equal to the lowest values for irradiated dogs.

The production of thrombocytes was surprisingly good by the dogs constantly exposed to levels of 17 R, 10 R, and 5 R/day (Figure 64). There was a well-expressed dose response during the first 160 days; from that time to 320 days the production by the 17 R and 10 R dogs was closely parallel. In the period from 320 to 570 days the values by 17 R, 10 R, and 5 R dogs have been parallel and steadily increasing so that they now average nearly 200,000 cells/mm³.

FIG. 63.—Effect of ^{60}Co gamma rays upon mean corpuscular volume (MCV) of erythrocytes and the number of reticulocytes in venous blood in beagles surviving 40 days exposure at 35 R/day, and in other groups exposed continuously at rates of 17 R and 10 R/day.

FIG. 64.—Effect of ^{60}Co gamma rays upon the number of venous thrombocytes in beagles at exposure rates of 5 R, 10 R, 17 R, and 35 R/day. One group was exposed to 35 R/day until death and the other terminated after 40 days.

The dogs exposed at the rate of 17 R/day have a highly individualistic response to protracted radiation judging from variation in subpopulations of venous leukocytes. A dog that died at 85 days had the lowest initial number of mature neutrophils ($5500/\text{mm}^3$) and an uninterrupted decline of all types of cells to death. The two dogs that died at 115 and 122 days had more mature neutrophils initially, abortive increases in these cells and eosinophils, and a somewhat better lymphocyte production. The two dogs that died at 135 and 170 days maintained mature neutrophil production at a higher level for a longer period; both started to produce large numbers of atypical lymphocytes around 90 days, and one had an early abortive eosinophilia. The dog that survived for 300 days started with less than 5000 mature neutrophils; these slowly dropped to 1000 at 115 days and remained at that

level until death; it had large numbers of atypical lymphocytes initially and persistently. The two dogs surviving through 590 days have persistently maintained comparatively high leukocyte numbers made up largely of mature and immature neutrophils.

CONCLUSION

Protracted exposure (590 days, 22 hr/day) of beagles to ^{60}Co radiation at rates of 35 R, 17 R, 10 R, and 5 R/day resulted in marked dose-responsive reduction in venous cell populations during the first 200 days. During the second half of the 590-day period the dose response effect disappeared; the dogs surviving at the 17 R, 10 R, and 5 R levels produced nearly equivalent numbers of leukocytes, erythrocytes, and thrombocytes in the venous blood. In the most interesting groups, the dogs at the 17 R/day

level, there is a persistent, comparatively high level of neutrophils.

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THE RESPONSE OF ANL BEAGLES TO PROTRACTED EXPOSURE TO ^{60}Co γ RAYS AT 5 TO 35 R/DAY. IV. BACTERIOLOGIC FINDINGS

Patricia C. Brennan and Richard C. Simkins

PURPOSE AND METHODS

We have continued to collect blood and tissues for culture from dogs exposed to continuous ^{60}Co radiation. The methods used were reported previously.⁽¹⁾

PROGRESS REPORT

All cultures of blood collected from dogs at the time their WBC count reached either approximately $5 \times 10^3/\text{mm}^3$ (stage 2) or $2.5 \times 10^3/\text{mm}^3$ (stage 3), have been negative. Pre-irradiation blood cultures (stage 1) were discontinued.

The bacteriologic findings on blood cultures collected when the WBC count fell below $1 \times 10^3/\text{mm}^3$ and/or the dog had a temperature $\geq 40^\circ\text{C}$ (stage 4), and on cultures from moribund or decedent animals (stage 5) are shown in Table 33. Dogs receiving 35 R/day generally died with bacteremia. One dog, No. 1603, irradiated at this dose rate, was not septicemic. Blood cultures collected from this dog when its temperature was elevated were negative, and blood cultures and tissue samples collected at necropsy failed to grow bacteria. Five more dogs at the 17 R/day exposure rate have died since our last report. Three of

these were septicemic; two were not. At the 10 R/day exposure rate, two of the nine dogs that died were septicemic, and at the 5 R/day exposure rate one of two that died was septicemic.

As before,⁽¹⁾ *Pasteurella multocida* and β -hemolytic streptococci were the usual causes of bacteremia. The recovery of *Escherichia coli* and *Clostridium perfringens* from the tissues of dead dogs may be the result of postmortem decomposition. We suggested that the β -hemolytic streptococci and *Pasteurella multocida* originate from the upper respiratory tract of the dogs, rather than the intestine.⁽¹⁾ This suggestion is strengthened by our finding that 41.8% of apparently healthy dogs carry β -hemolytic streptococci in their throats, and 79.5% carry *P. multocida*.⁽²⁾

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TABLE 33. BACTERIAL FINDINGS IN BEAGLE DOGS GIVEN CONTINUOUS EXPOSURE TO ^{60}Co γ RADIATION

Dose, R/day	Dog No.	Specimen cultured	Stage ^(a)	Bacteria recovered					
				β -hemo- lytic strep- tococci	<i>Pasteurella</i> <i>multocida</i>	<i>Clostridium</i> <i>perfringens</i>	<i>Escherichia</i> <i>coli</i>	<i>Staphy-</i> <i>lococcus</i> <i>aureus</i>	Other
35	1543	Blood Blood Tissues	4		No positive cultures				
			5	+		+			
35	1557	Blood Blood Tissues	4		No positive cultures				
			5	+			+		
35	1603	Throat Blood Blood Tissues	4		+		+		
			5			No positive cultures			
35	1629	Blood Blood Tissues	4			No positive cultures			
			5	+		No positive cultures			
35	1632	Throat Blood Blood Tissues	4			No pathogens recovered			
			5	+					
35	1633	Blood Blood Tissues	4		No positive cultures				
			5	+		+	+		
35	1665	Blood Blood Tissues	4	+					
			5	+			+		<i>Bacteroides</i> sp.
35	1683	Blood Tissues	5		+	+			
					+	+			
35	1687	Blood Tissues	5			+	+		
						+	+		
35	1533	Blood Tissues			+	+			
					+	+			
35	1547	Blood Tissues	5		+				
						+		+	
35	1556	Blood Tissues	5			+	+		
					+	+	+		
35	1688	Blood Tissues	5		+	+			
					+	+			
35	1721	Blood Blood Tissues	4		No positive cultures				
			5	+			+		
35	1714	Blood Tissues	5		+	+	+		
					+	+	+		
35	1723	Blood Blood Tissues	4						<i>Aspergillus</i> <i>ustus</i> ^(b)
			5	+	+	+			<i>Aspergillus</i> <i>ustus</i> ^(b)
35	1700	Blood Tissues	5		+				α streptococci
					+			+	α streptococci

TABLE 33- Continued

Dose, R day	Dog No	Specimen cultured	Stage ^{a)}	Bacteria recovered					Other	
				β -hemo- lytic strep cocci	<i>Pasteurella multocida</i>	<i>Clostridium perfringens</i>	<i>Escherichia coli</i>	<i>Staphy- lococcus aureus</i>		
35	1709	Blood Tissues	5	+	+					
17	1209	Blood Tissues	4			No positive cultures + (PMD) ^e				
17	1502	Blood Blood Tissues	4 5			+		+		
17	1523	Blood Tissues	5				+	+		
17	1542	Blood Tissues	5	+					+	
17	1581	Blood Tissues	5			No pathogens recovered No pathogens recovered				
10	1348	Blood Blood Tissues	4 5			No positive cultures				
10	1351	Blood Blood Tissues	4 5	+				+	<i>Alcaligenes</i> sp	
10	1351	Blood Blood Tissues	4 5			No pathogens recovered No pathogens recovered				<i>Bacteroides</i> sp (tran- sient)
10	1397	Blood Tissues	5			No positive cultures No pathogens recovered				
10	1467	Blood Blood Tissues	4 5			No positive cultures No positive cultures No growth				
10	1341	Blood Blood Tissues	4 5		+	No positive cultures No positive cultures				<i>Alcaligenes</i> sp
10	1362	Blood Tissues	4			No positive cultures No growth				
10	1434	Blood Tissues	5			No positive cultures + (PMD)				
10	1462	Tissues				No pathogens recovered				
10	1474	Blood Tissues	5			No positive cultures No growth				
5	1361	Blood Blood Tissues	4 5			No positive cultures No positive cultures + (PMD)				
5	1334	Blood Blood Tissues	4 5			+		+		
						+	+	+		

^(a) Stage 1 Prenradiation blood culture Stage 2 Blood culture taken when the WBC was about $5 \times 10^3/\text{mm}^3$ Stage 3 Blood cultures taken when the WBC was about $2.5 \times 10^3/\text{mm}^3$ Stage 4 Blood cultures taken when the WBC was less than $1 \times 10^3/\text{mm}^3$ and/or the temperature was 40°C Stage 5 Blood cultures taken when the dog was moribund or dead

^(b) Associated with decomposing organic matter

^(c) Postmortem decomposition

THE RESPONSE OF ANL BEAGLES TO PROTRACTED EXPOSURE TO ^{60}Co γ RAYS AT 5 TO 35 R DAY. V. PATHOLOGY

Thomas E. Fritz, Ruth C. Zeman, David V. Tolle, and John W. Williams

PURPOSE AND METHODS

During the past year, we have continued examinations and collection of specimens and data according to protocols described previously.⁽¹⁾

PROGRESS REPORT

The death of dogs in the experiment during the past year has supported our previous prognosis, that main causes of death after protracted irradiation would fall into two categories: septicemia and anemia.⁽¹⁾ Septicemia is the main cause of the earlier deaths which occur at the higher doses, while anemia accounts for deaths at longer times in the lower dose groups. The incidence of septicemic deaths is discussed in another portion of this report.⁽²⁾

The most important finding during the past year has been two cases of myeloproliferative disease in dogs irradiated at 10 R/day. These dogs died after 383 and 504 days of γ -ray exposure. The lesions were similar in both dogs, and included splenomegaly, hemorrhagic lymphadenopathy, and edema of various tissues, including the lungs and body cavities. There were also scattered petechial and ecchymotic hemorrhages of the gastrointestinal tract. Both decedents showed a progressive anemia before death but had neither leukemia nor leukocytosis. Preliminary examination of post-mortem marrow samples revealed

a marked increase in the juvenile, or undifferentiated, forms of the myeloid elements. The microscopic tissue changes in these two dogs included proliferation of juvenile or undifferentiated myeloid cells in the liver, spleen, and lymph nodes. These histopathologic changes are similar to those previously described in dogs with developing myeloproliferative disorders after intravenous single doses of ^{144}Ce or protracted whole-body irradiation given for 100 days.^(3, 4)

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THE DEPENDENCE OF ^{137}Cs RETENTION ON AGE IN THE JUVENILE BEAGLE DOG

Sylvanus A. Tyler, Norbert D. Kretz, and William P. Norris

PURPOSE AND METHODS

The retention of ^{137}Cs by various mammals is reported to be related to body size.⁽¹⁾ Other studies⁽²⁾ of ^{137}Cs retention lead to the conclusion that young mice excrete ^{137}Cs faster than old mice. We have investigated this dependency on age by selecting, as experimental subjects, young, growing dogs from our colony of beagles. Our specific objective is to determine and quantitatively assess the influence of the gross weight of the dog on the retention of ^{137}Cs . Then we propose to attempt an answer to the question: which is the dominant factor in the retention of ^{137}Cs —age or body weight?

Cesium-137 retention at various times after injection was determined for individual dogs ranging from 61 to

588 days of age at injection. These determinations were made by periodic measurements of whole-body gamma-ray activity.

PROGRESS REPORT

The retention of ^{137}Cs has been followed in dogs injected at 13 different ages. Representative portions of this data are presented in Figure 65. These data show 1) that the specific rate of change in retention, $(1/R) \cdot (dR/dt)$, decreases in absolute value as the age of the dog increases; and 2) that the specific rate of change in ^{137}Cs retention approaches a constant value (p) as the dog reaches maturity.

It is known from descriptions of growth based on the Gompertz function as a model:⁽³⁾ that 1) the specific

rate of change in weight, $(1/W) \cdot (dW/dt)$, decreases as the age of the animal increases and 2) the specific rate of change in weight approaches zero as the animal reaches maturity. These qualitative similarities led to the development of a model⁽⁴⁾ for ^{137}Cs retention based on the Gompertz function. The retention of ^{137}Cs in a dog injected at age a , $R_{a,t}$, is mathematically expressed as

$$R_{a,t} = R_{a,0} \text{Exp} \left\{ -pt - \frac{A_{a,0}}{\alpha} [1 - \text{Exp}(-\alpha t)] \right\}, \quad (1)$$

where $A_{a,0} + p = (1/R) \cdot (dR/dt)$, the specific rate of change in retention at time of injection;

p = The constant value to which the specific rate of change in retention decays;

$A_{a,0}$ = That part of the specific rate of change in retention at injection which is decaying;

α = The constant, proportional rate at which $A_{a,0}$ is decaying with age;

$R_{a,0}$ = Scale factor;

t = Time after injection.

As stated, the rate p is approached when the dog reaches maturity. From a practical point of view, maturity is reached at about 300 days of age. The retention data post-300 days of age was fit with a single exponential function and the rate constant p was determined. With p determined, the parameters $A_{a,0}$, α , and $R_{a,0}$ of Equation (1) were estimated by an iterative least squares procedure for the retention data at each age. The compatibility of the model with retention data is exhibited in Figure 65.

With a mathematical description of ^{137}Cs retention in young growing dogs, it is possible to determine whether growth correlates with ^{137}Cs retention. In a previous report by Tyler and Norris,⁽⁵⁾ the Gompertz function was used to describe the accretion of weight by growing dogs in this colony. The model used for this analysis is mathematically expressed as:

$$W = W_0 \text{Exp} \left[\frac{A_0}{\alpha} [1 - \text{Exp}(-\alpha t)] \right], \quad (2)$$

where W = Body weight,

W_0 = Body weight at birth,

$A_0 = (1/W) \cdot (dW/dt) |_0$, the specific rate of change in weight at birth,

α = The constant proportional rate at which A_0 is decaying with age,

t = The age of the animal.

FIG. 65.—Retention of ^{137}Cs administered by intravenous injection to beagles of different ages. Curves are drawn based on Equation (1) and the estimated values of the constants.

A comparison of the values of the ^{137}Cs retention parameters with the values of the corresponding growth parameters shows that the value of α for ^{137}Cs retention is the same as the value of α for growth. The comparison also shows that the values for $A_{a,0}$ and A_a maintain a constant ratio at all ages.

CONCLUSION

Constants of the model, derived from ^{137}Cs retention, reveal that the specific rate of change of retention is a continuously decreasing function of age and is not influenced by the particular age at injection. Comparison of the retention model and the growth model demonstrates the high degree of correlation between ^{137}Cs retention and growth, expressed in terms of body weight.

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Characterization of the ANL Beagle

STUDIES OF THE PHYSIOLOGY OF THE BEAGLE THYROID GLAND. INTRODUCTION

William P. Norris

Before studying the response of the beagle thyroid gland to ionizing radiation, we have made preliminary explorations of the physiologic properties of the gland. This work has resulted in certain observations and conclusions, either new or previously only partially understood:

1. The beagle thyroid is highly responsive to changes in dietary iodine. Such response is accompanied by large changes in all aspects of ^{131}I metabolism by the thyroid. The response may proceed for as long as one year before a new steady state is reached.
2. Lymphocytic thyroiditis is an inherited disease

of the thyroid in the beagle, much as it seems to be in man.

3. Tracer studies using ^{131}I allow nondestructive identification of colony beagles with and without lymphocytic thyroiditis.

This work is described in more detail in the three reports that follow. Because they identify a large, normal responsiveness to a simple stimulus—changes in dietary iodine—they provide a strong basis for further study of thyroid physiology and also for work with the irradiated thyroid gland. Further, they allow the work to be done using dogs with either normal or abnormal thyroid tissue.

STUDIES OF THE PHYSIOLOGY OF THE BEAGLE THYROID GLAND. I. THE CYCLE OF ACCOMMODATION TO RESTRICTED DIETARY IODINE IN THE THYROID GLAND OF THE ANL BEAGLE DOG

William P. Norris, Thomas E. Fritz, and James A. Taylor^{*}

PURPOSE AND METHODS

Published recommendations for adequate dietary allowances in dogs⁽¹⁾ include, seemingly, unnecessarily large amounts of iodine. Because such recommendations influence the composition of commercial dog foods, the average dog maintained on commercial diets exhibits a very low (~ 1 to 5%) thyroidal uptake of test doses of ^{131}I . Such low thyroidal uptake makes certain experimental work with ^{131}I difficult or impractical.

Young adult beagles, previously fed a commercial diet estimated to provide at least $500 \mu\text{g I}^-/\text{dog}/\text{day}$, were switched to a semi-synthetic diet (made of casein, sucrose, cotton seed oil, and purified vitamins and minerals) containing $25 \mu\text{g I}^-/100 \text{ g}$. Measurements of food intake showed the total dietary intake of iodide to range from 50 to $75 \mu\text{g}/\text{dog}/\text{day}$.

These dogs were given test doses of ^{131}I at intervals over a period of 651 days to measure thyroidal uptake of ^{131}I and its subsequent rate of loss from the gland. After these measurements, the dogs were killed serially to examine thyroid, pituitary, and adrenal tissue. Weights of these endocrine glands were re-

corded. Results of these observations were compared with similar data from dogs in the regular colony eating either the standard commercial diet or the same semi-synthetic diet containing I^- equivalent to a dietary intake of $450 \mu\text{g I}^-/\text{dog}/\text{day}$.

PROGRESS REPORT

The microscopic appearance of the normal beagle thyroid gland is shown in Figure 66. During the first 268 days of restricted iodide intake, the thyroid glands became increasingly hyperplastic and hypertrophic (Figure 67). Hyperplasia and hypertrophy were correlated with a large increase in the thyroidal uptake of test doses of ^{131}I . During the period of hyperplasia, the rate of loss of ^{131}I from the thyroid gland became increasingly more rapid. By 268 days after initiation of the low iodide diet, the biological half-time ($\text{BT}_{1/2}$) of iodine in the thyroid gland had decreased to 3.9 days from the pre-experimental value of 12.5 days. These data are presented in Table 34 and graphically demonstrated in Figure 68.

After 368 days of restricted iodide intake, the thyroid glands were involuted and had an essentially normal histologic appearance. The size and weight of the thyroids were, by this time, about three times

^{*} Chas. Pfizer & Co., Groton, Conn

FIG. 66. —Typical follicular structure and parafollicular cell population (arrow) in the thyroid gland of dogs fed the commercial ration. Hematoxylin and eosin stain.

FIG. 67. —Thyroid gland of a beagle dog given the low-iodide diet for 268 days. Nearly complete loss of colloid and severe folding of the follicular epithelium are shown. Hematoxylin and eosin stain.

TABLE 31. SUMMARY OF WEIGHTS OF ENDOCRINE GLANDS AND OF UPTAKE AND RELEASE OF ^{131}I BY THYROID GLANDS OF BEAGLE DOGS FED RESTRICTED AMOUNTS OF IODIDE

Time fed low-iodide diet, days	Endocrine gland weights					Uptake and release of ^{131}I		
	No. of dogs	Adrenal glands, g $\pm$ S.E.M.	Pituitary gland, mg $\pm$ S.E.M.	Thyroid gland, g $\pm$ S.E.M.	Weight of thyroid gland/body weight $\times 10^4 \pm$ S.E.M.	No. of dogs	Maximal thyroid ^{131}I uptake, ^(a) % $\pm$ S.E.M.	BT _{1/2} , ^(b) days $\pm$ S.E.M.
0	17 ^(c)	1.16 $\pm$ 0.06	61.8 $\pm$ 5.7	0.98 $\pm$ 0.07	0.67 $\pm$ 0.03	—	—	—
0	—	—	—	—	—	11 ^(c)	11.88 $\pm$ 1.08	12.53 $\pm$ 1.06
48	—	—	—	—	—	6	22.1 $\pm$ 2.3	8.2 $\pm$ 1.3
75	6 ^(d)	1.28 $\pm$ 0.08	92.7 $\pm$ 8.9	0.88 $\pm$ 0.10	0.93 $\pm$ 0.14	—	—	—
118	1	1.45	81	1.90	1.84	6	46.4 $\pm$ 5.1	7.2 $\pm$ 1.5
182	1	1.70	88	2.65	2.58	5	52.6 $\pm$ 6.5	5.8 $\pm$ 1.4
268	1	1.70	67	3.05	3.10	4	60.2 $\pm$ 8.8	3.9 $\pm$ 1.1
368	1	1.20	65	1.62	1.58	3	65.0 $\pm$ 9.0	25.7 $\pm$ 4.5
453	1	1.20	85	2.95	2.41	2	66.2 $\pm$ 10.1	32.5 $\pm$ 2.5
553	—	—	—	—	—	1	65.0	36.0
651	1	1.40	80	3.80	2.96	1	72.0	22.6
Mean for 6 serially killed dogs (days 118, 182, 268, 368, 453 and 651)		1.44 $\pm$ 0.09	77.7 $\pm$ 3.9	2.66 $\pm$ 0.32	2.41 $\pm$ 0.25			

^(a) Where dogs were serially killed, ^{131}I for this test was administered 14 days before they were killed.

^(b) Biological half-time of iodine in thyroid glands computed from serial measurements of ^{131}I in the gland between 1 and 11 days after ^{131}I was intravenously administered.

^(c) These data are from untreated dogs in this colony fed the commercial ration. Weights of endocrine glands were from 17 dogs averaging 475 (between 322 and 559) days of age. See Reference 2 for ^{131}I uptake data in colony dogs.

^(d) These dogs were totally thyroidectomized after being given the diet with restricted iodide content for 75 days.

normal. No changes in the pituitary or adrenal glands were noted. Thyroid involution correlated with an abrupt increase in the BT_{1/2} of iodine in the gland to about 30 days.

This is the first report of the experimental demonstration of the complete cycle of thyroid accommodation to reduced iodine intake. During this cycle, there are major changes in all aspects of thyroidal iodine metabolism measured by test doses of ^{131}I , as well as in histologic structure of the gland. In this instance the cycle was completed in about one year.

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FIG. 68. Progressive changes in ^{131}I metabolism of an 11-month-old beagle dog after a large reduction in the iodide content of the diet. Measurements are of the *in vivo* content of the thyroid gland vs. time after intravenous administration of tracer doses. Repetitive tests were initiated in the same dog after 48, 104, 168, 254, 354, 439, 539, and 637 days. Results for 439, 539, and 637 days were very similar to those for 354 days and are omitted for the sake of clarity.

STUDIES OF THE PHYSIOLOGY OF THE BEAGLE THYROID GLAND. II. PATHOLOGY AND FAMILIAL INCIDENCE OF THYROIDITIS IN A CLOSED BEAGLE COLONY

Thomas E. Fritz, Ruth C. Zeman, and Max R. Zelle

PURPOSE AND METHODS

The occurrence of thyroiditis in the Argonne beagle colony has been reported.^(1,2) During the past year, our efforts to define the incidence, pathology, and heredity aspects of the disease have continued. Specimens of thyroid tissue from 401 dogs of all ages and their pedigrees have been examined.

PROGRESS REPORT AND CONCLUSIONS

Thyroiditis occurs spontaneously in the colony with an incidence approaching 20% in untreated animals over one year of age (Table 35). The disease in dogs over 400 days of age is genetically influenced as determined by Chi-square tests of the ancestral composition of each animal (Table 36). The incidence increases with increasing degrees of relatedness to three sibling progenitors of a partially inbred line, A (Table 36), which comprises a major portion of the colony. The pathologic changes in the thyroids cause neither clinical signs nor enlargement of the gland, and the severity of the lesions is not related to age. The lesions do not appear to be progressive in severity or to result in atrophy or fibrosis of the gland. Although

the histologic features and total incidence of thyroiditis in adult dogs given lethal or near-lethal doses of ionizing radiation is distinctly different from thyroiditis in unirradiated dogs (Table 35), its incidence is genetically influenced in the same manner as in unirradiated adults (Table 36). Lymphocytic lesions also occur in the thyroids of 55% of stillborn puppies (Table 35), but are different from those in adult dogs. In stillborn puppies, the incidence is not genetically influenced as it is in the adult dogs.

A report describing this work is in press.⁽³⁾

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TABLE 35. INCIDENCE OF THYROIDITIS IN 401 BEAGLES BY EXPERIMENTAL STATUS, AGE, AND SEX

Group	Status	Age, days	Incidence					P ^(a) for groups compared				
			Male	Female	P ^(a)	Total		1	2	3	4	5
1	Nonexperimental	0 (still born)	11/19 (57.8%) ^(b)	9/17 (52.9%)	>0.05	20/36 (55.5%)	-	<0.01	<0.01	<0.01	<0.01	<0.01
2	Nonexperimental	1-10	0/24 (0%) ^(c)	2/28 (7.1%)	>0.05	2/52 (3.8%)	<0.01		>0.05	<0.01	>0.05	
3	Nonexperimental	11-400	0/14 (0%)	0/11 (0%)	>0.05	0/25 (0%)	<0.01	>0.05		<0.01	<0.01	
4	Nonexperimental	401+	11/63 (15.8%) ^(d)	16/73 (21.9%)	>0.05	27/136 (19.8%)	<0.01	<0.01	<0.01			<0.01
5	Experimental	401+	8/80 (10.0%) ^(e)	6/72 (8.3%)	>0.05	14/152 (9.2%)	<0.01	>0.05	<0.01	<0.01		-

^(a) Significance determined by computing a *t* value from standard error of the difference of two proportions being compared.

^(b) No. positive No. examined.

^(c) For statistical analysis 0 incidence was replaced by 0.5.

^(d) Mean $\pm$ S.E. = 1306 ± 82

^(e) Mean $\pm$ S.E. = 832 ± 55 ; $t_{256 \text{ D.F.}} = 4.73$; $P < 0.01$.

TABLE 36 INCIDENCE OF THYROIDITIS BY ANCESTRAL COMPOSITION

ϵ_c line A	No. positive/ No. examined	ϵ_c positive	ϵ_c line B	No. positive/ No. examined	ϵ_c positive
Group 1 -Nonexperimental Stillborn					
0	1/4 ^a	25 0	0	7/15	46 7
12 5	0/2	0	25 49	11/14	78 6
25	9/11	81 8	50 74	1/4	25 0
50	5/7	71 4	100	1 3	33 3
100	5/12	41 7			
Total	20/36	55 6	Total	20/36	55 6
Group 2 Nonexperimental 1 to 10 days of age					
0	0/3	0	0	1/37	2 7
25	1/12	8 3	1 24	0 3	0
100	1/37	2 7	25 49	1/10	10 0
			50 74	0 1	0
			100	0/1	0
Total	2/52	3 8	Total	2/52	3 8
Group 3 Nonexperimental 11 to 400 days of age					
0	0/6	0	0	0/6	0
12 5	0/2	0	1 24	0/4	0
25	0/8	0	25 49	0 9	0
50	0 6	0	50 74	0 6	0
100	0 3	0			
Total	0/25	0	Total	0/25	0
ϵ_c line A	No. positive/ No. examined	ϵ_c positive	ϵ_c line B	No. positive/ No. examined	ϵ_c positive
Group 4 Nonexperimental 401+ days of age					
0	1/52	1 9	0	17/45	37 8
25	4/31	12 9	1 24	2 24	8 3
50	5/18	27 8	25 49	3/38	7 9
62 5	0/1	0	50 74	4/22	18 2
100	17/34	50 0	100	1/7	14 3
Total	27/136	49 9	Total	27/136	49 9
Group 5 Experimental dogs -401+ days of age					
0	2/72	2 8	0	7/53	13 2
25	1/22	4 5	1-24	3 29	10 3
50	3/18	16 7	25 49	2 33	6 1
75	2/11	14 3	50-74	1 31	3 2
100	6/26	23 1	75	0/3	0
			100	1 3	33 3
Total	14/152	9 2	Total	14/152	9 2

^a Eleven dogs were between 345 and 397 days of age

STUDIES OF THE PHYSIOLOGY OF THE BEAGLE THYROID GLAND. III. INFLUENCE OF THYROIDITIS ON BIOLOGICAL HALF-TIME OF ¹³¹I IN THE ANL BEAGLE DOG

Thomas E. Fritz, William P. Norris, Norbert D. Kretz,
Ruth C. Zeman, and John W. Williams

PURPOSE AND METHODS

Thyroiditis, recognized as a disease entity in our beagle colony, has been studied in this laboratory for two years.^(1,2) Because this disease produces no clinical signs, a nondestructive method of diagnosis is highly desirable. This subject was treated in last year's report.⁽²⁾

Six dogs were selected for examination on the basis of their pedigrees as being likely candidates for thyroiditis and were injected with 20 μ Ci of ¹³¹I. Their thyroid activity was measured for 30 days, or until it could no longer be detected.

Similarly, the six dogs reported last year, each of which had had one thyroid removed 13 months earlier,

were also injected with 20 μ Ci of ¹³¹I, and the thyroid activity was followed. At the end of the 30-day period, one thyroid gland was removed from each of the 12 dogs; in the case of the second 6, the second lobe was removed. Sections were prepared by the paraffin method for microscopic examination. When changes of thyroiditis were observed they were scored as to their severity according to the method of Beierwaltes and Nishiyama.⁽³⁾

PROGRESS REPORT

Table 37 summarizes the results of tests conducted on the twelve dogs. The results of the first test on 6 of the dogs were reported last year,⁽²⁾ but are included

TABLE 37. DIAGNOSIS OF THYROIDITIS IN BEAGLES

Dog No.	Sex	Age	Thyroid weight, g	Histology ^(*)	Max. uptake, % inj. dose	BT _{1/2} , days
First ¹³¹ I tracer test (left thyroid gland)						
0193	M	2778	0.487	0	1.8	13.0
0215	M	2759	0.491	0	2.0	13.6
0231	M	3151	0.200	3+		1.2
0306	F	3000	0.370	3+	0.4	4.0
0403	M	2780	0.432	0	1.8	7.8
0495	F	2572	1.800	3+	0.9	4.4
1084	F	1078	0.520	3+	1.4	3.0
1129	F	1043	0.460	3+	0.7	5.4
1131	M	1043	0.520	3+	0.2	3.4
1176	F	1397	0.241	3+	0.4	2.0
1231	M	852	0.476	0	2.0	14.0
1719	M	580	0.370	0	1.5	14.2
Second ¹³¹ I tracer test initiated 400 days after hemithyroidectomy (right thyroid gland)						
0193	M	3205	0.375	1+	1.6	8.0
0215	M	3186	0.369	1+	1.3	6.0
1084	F	1505	0.494	3+	0.2	3.0
1129	F	1170	2.560	3+	0.8	2.0
1131	M	1470	0.454	3+	0.2	2.8
1231	M	1279	0.455	0	1.2	8.3

(*) 0 = no evidence of disease;

1+ = focal lymphocytic infiltrate(s) with no epithelial involvement;

2+ = grade 1+, plus epithelial involvement, i.e., acinar atrophy, disintegration of follicular cells and presence of macrophages;

3+ = grade 2+, plus Hurthle cell changes of the follicular epithelium.

here for purposes of comparison with this year's dogs. The results of the first test of all 12 dogs support our conclusion of last year. The rate at which the thyroids lost ¹³¹I after the point of maximum uptake is diagnostic for thyroiditis. The mean BT_{1/2} (biological half-time) for dogs without thyroiditis was 12.5 days $\pm$ S.E. = 1.19, and that of dogs with thyroiditis was 3.34 ± 0.54 ($t_{(11)}^* = 7.74$; $P < 0.005$).

Although the BT_{1/2} in this instance does relate to thyroiditis, it is more valid to say the BT_{1/2} correlates with the presence or absence of normal thyroid follicular structure and the amount of colloid. This is

* (11) = degrees of freedom.

demonstrated by the results of the second test of 6 dogs which previously had been tested and had one gland removed. Two of these dogs were considered histologically negative after the first test, but positive (1+) after the second test. Even though they had mild thyroiditis, the BT_{1/2} was similar to that in dogs without thyroiditis. Dogs without thyroiditis and dogs with mild (1+) thyroiditis have glands that are histologically similar, except for the presence of small aggregations of lymphocytes in the interstitial spaces. Thyroids with severe thyroiditis (3+), on the other hand, consisted largely of nonfollicular sheets of parafollicular-type cells, and colloid is greatly reduced or, in some, virtually absent.

To verify further the relationship between relative proportions of colloid and epithelial components and the BT_{1/2} of ¹³¹I in the gland we intend to use the technique of Uotila and Kannas⁽⁴⁾ to quantitate the proportions of the components of the thyroid gland.

CONCLUSION

The biologic half-time (BT_{1/2}) of ¹³¹I in the thyroid, determined by calculating the rate at which ¹³¹I activity decreases following maximum uptake, is a practical method for establishing the presence of thyroid lesions in a beagle colony with a high incidence of thyroiditis. Dogs with severe thyroiditis have a significantly shortened BT_{1/2} compared to dogs with no thyroiditis or dogs with mild lymphoid cell infiltration of the gland. This test can be used without rigidly controlling the dietary iodide intake.

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SKIN GRAFT SURVIVAL IN PARTIALLY INBRED BEAGLES*

Carl E. Rehfeld, Gustave J. Dammin,^{††} and William J. Hester^{†*}

Skin grafts were exchanged between members of 22 pairs of inbred and cousin-related beagles. The average time from implantation to slough in allograft controls was 11.7 days and ranged from 9 to 48 days for all other allografts. Eleven allografts survived 22 or more days, suggesting a donor-recipient relationship which approximates that between fraternal twins. All grafts were observed for contraction, healing, three phases of

rejection, and mode of rejection; these factors were correlated with coefficients of inbreeding and relationship. Graft contraction appeared to have some predictive value, because if grafts continued to contract after 17 days they did eventually slough. The rate and degree of healing at incised edges of donor and recipient skin appeared to be a sensitive indicator of histocompatibility. Grafts that sloughed before 17 days were usually intact, in the sense that the epidermal, and perhaps the dermal, layers were still present. Grafts that survived more than 17 days usually sloughed by lytic action. There was a better correlation between coefficient of relationship and graft survival than there was for the inbreeding coefficient and survival.

* Abstract of a paper being prepared for publication.

†† This is a collaborative study between Argonne National Laboratory and the Department of Pathology, Harvard Medical School, Peter Bent Brigham Hospital, Boston, Massachusetts.

DEFINITION OF RELATIONSHIPS IN A CLOSED BEAGLE COLONY*

Carl E. Rehfeld

A comprehensive program is described for definition of inbreeding, relationships, and ancestral composition within an animal population. Inbreeding coefficients may be used to evaluate breeding history and to control inbreeding levels. Coefficients of relationship are most useful for accurate pairing of animals with respect to familial relationships. Ancestral composition may be used to define familial subpopulations within a colony so that inherited physiological and biochemical variations may be included in the evaluation of experimentally produced changes. A closed beagle colony of 1690 dogs is used to illustrate both the application and value of the three criteria

of breeding and relationship. Twelve hundred and one (71.1%) of these dogs have some degree of inbreeding. The coefficient of relationship is dependent upon how closely animals are related, a value that may be enhanced if the dogs are inbred; it is a means of selecting pairs with equivalent relationship for histocompatibility and other studies where accurate pairing is important. Ancestral composition defines the two inbred subpopulations within this colony and the extent of their outbreeding with the other two subpopulations. Neonatal survival is used to illustrate inbreeding effects, and the effects of outbreeding between the subpopulations are assessed by weanling survival.

* Abstract of paper to be published in the *Am. J. Vet. Res.*

THE ULTRASTRUCTURE OF NORMAL HEMOPOIETIC CELLS FROM DOG BONE MARROW

Theodore N. Tahmisian, Rosemarie L. Derinc, Betty Jean Wright, William P. Norris,
Thomas E. Fritz, Ruth C. Zeman and David V. Toll

PURPOSE AND METHODS

Dependent upon dose rate, acute and chronic irradiation by γ rays from a cobalt source reveals a differential susceptibility of hemopoietic cells in the bone marrow of dogs. In another section of this report,⁽¹⁾ it is demonstrated that bone marrow cell pro-

liferation in the beagle dog essentially ceases when the animals are subjected to continuous exposure to ^{60}Co γ radiation at 35 R/day. At half this exposure rate, 17 R/day, or below, however, marrow cells multiply successfully, and animals may survive for extended periods of time. It is desirable, therefore, to

FIG. 69 Plasma cell with extensive endoplasmic reticulum.

FIG. 71 An osteoblast with collagen fibers

FIG. 70 Megakaryocyte, which forms blood platelets

examine the ultra-structure of marrow cells from dogs given continuous irradiation at these exposure rates that are critical for continued marrow cell survival. As a first step, it became necessary to examine and identify normal marrow cells for comparison at a future time with similar irradiated cells.

We present the ultra-structure of the various types of normal bone marrow cells identified with the aid of the electron microscope. The results may be compared with the work of Bessis.⁽²⁾

Immediately following euthanasia, bone marrow was removed from the femur of a beagle and fixed in Karnovsky's⁽³⁾ glutaraldehyde-paraformaldehyde solution buffered with phosphate. The tissues were fixed at 0 to 5 C° for 3 to 5 hr, then washed overnight with several changes of phosphate buffered saline and post-fixed for 1 hr at 0 to 5 C° in 1% OsO_4 solution phosphate buffered to pH 7.4. They were then dehydrated with alcohols, followed by propylene oxide, and embedded in epoxy resins as described by Luft.⁽⁴⁾

The blocks were sectioned with a Reichert Om U2 ultramicrotome. The sections were stained with uranyl acetate and lead citrate as described by Ven-

FIG. 72. - Collagen fibers in longitudinal and cross section.

FIG. 73. - Mature neutrophil at upper right, portion of a red blood corpuscle at left center, and an eosinophilic myelocyte in metaphase. This cell shows cytoplasmic granules within which crystals are formed.

FIG. 74. Reticular cell upper left, two metarubricytes upper and central right and two neutrophilic myelocytes at the lower end of the plate.

FIG. 75. Macrophage with ingested debris and portion of a reticular cell at lower left.

FIG. 77. Neutrophilic myelocyte with unsegmented nucleus.

FIG. 76. Neutrophilic promyelocyte.

FIG. 78. Mature neutrophilic granulocyte with segmented nucleus (lower left) and portions of red blood corpuscles at the upper portion of the plate.

ble et al.⁽⁵⁾ and observed with the aid of a Siemens Elmiskop 1A. Photomicrographs were obtained at a constant original magnification of 6,000 $\times$, except where large cells such as megakaryocytes required a lower magnification to include the whole cell in the photographic plate. A point source Durst enlarger was used for printing the electron photomicrographs.

PROGRESS REPORT

In this preliminary work we will only identify the different types of cells found in the bone marrow. Figure 69 shows a plasma cell with its typical branched endoplasmic reticulum. Figure 70 shows a mature, platelet-forming megakaryocyte. In order to include most of the cell on one plate it was photographed at a low magnification. Cytoplasmic detail is not well represented; azurophilic granules and mitochondria are easily visible when higher magnification photographs are obtained. Figure 71 shows collagen fibers, an osteoblast, some bone and the brush border between the bone and cell tissue. Figure 72 shows some collagen fibers. Figure 73 shows a mature neutrophil,

an eosinophilic myelocyte in mitosis (note granules with "crystals"), and portions of red blood corpuscles. Figure 74 shows two metarubricytes, two neutrophilic myelocytes and a reticular cell. Figure 75 shows a macrophage with ingested debris and a reticular cell. Figures 76, 77, and 78 show developmental stages of neutrophilic granulocytes.

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MECHANISMS OF CARCINOGENESIS

Austin M. Bruce, Harry Auerbach, Georgia M. DeRoche, and Donald D. Grube

PURPOSES AND METHODS

This project has been concerned with the mechanisms of tumor induction by local irradiation of tissues and by the implantation of nonradioactive plastics and other foreign materials. The histologic response that precedes the induction of tumors by superficial irradiation of the skin are being studied through the changes in cell dynamics of the exposed epidermal populations. The method of double-isotope autoradiography has been modified to permit simultaneous determinations of the rates of cells flowing into and out of DNA synthesis. The use of chemical co-carcinogens and tumor promoters after local irradiation of the skin allow an assessment of the efficacy and nature of irradiation as an inducing agent in tumor production. Interspecies comparisons of these effects are also under study. Previous reports⁽¹⁻³⁾ have dealt with a number of these problems and include details of some of the methods employed. The methods devised for cell kinetics studies are described under the appropriate section. This report details progress on: 1) cell kinetics studies on mouse epidermis; 2) preliminary results of attempts to demonstrate promotion of skin carcinogenesis by croton oil extracts after external beta irradiation; 3) a study to determine whether castration changes the distribution of subcutaneously injected plutonium-239, and its relation to the production of bone tumors; 4) the action of implants containing radioactive material in dogs and 5) nonradioactive materials being tested for tumor production. Conclusions are included with each project progress statement.

PROGRESS REPORT

Cell Kinetic Studies on Mouse Epidermis

Deoxyribonucleic acid synthesis in the dorsal epidermis of the hairless mouse, H₁/Anl[Anl 66], was studied during different periods of the day using labeled thymidine. The *S*-indices for the interfollicular areas of mouse epidermis, illustrated in Figure 79, follow a predictable day-to-day diurnal rhythm and are not unlike those reported by others.⁽⁴⁻⁵⁾ This

apparent change in the number of cells in DNA synthesis over a 24-hr period could be the result of any of the following: 1) a continuous change in the duration of the DNA synthesis (*S*) period; 2) a changing influx of cells into the *S*-compartment of the mitotic cycle; or 3) changes in both parameters. For use in clarifying this problem, we have been looking critically at the classical method of double-label autoradiography.⁽⁶⁾ Theoretically, autoradiography after separate injections of differently labeled thymidine should make it possible to estimate, simultaneously, changes in the rates at which proliferating cells move into and through the compartments of the mitotic cycle. Usual methods do not differentiate between nuclei labeled with carbon-14 and those which have incorporated thymidine labeled with both carbon-14 and tritium. Therefore, at best, the methods can give a relative measurement of only one flow parameter, i.e., the flow rate of cells either into or out of the *S*-compartment, and will not simultaneously show both rates in the same experiment. Determinations of the duration of DNA synthesis by such methods are valid only with constant flow rates in the cell cycle. Because, as indicated above, the diurnal cycle may result from changes in flow rate, the methods do not serve to investigate this possibility.

A modified autoradiographic technique,⁽⁷⁾ first described by Field et al.⁽⁸⁾ makes it possible to distinguish between nuclei labeled with tritium or carbon-14 by the use of two overlying emulsions. In the first the grains are dye-coupled, and in the second silver grains are seen. This has allowed us to measure, simultaneously, the relative flux of cells into and out of the *S*-compartment, using the same animal during an identical period of the diurnal cycle. The distinction made between nuclei labeled with one or both isotopes clearly demonstrated for the first time an inequality in the rate of passage of cells into and out of DNA synthesis. Table 38 shows two periods of the diurnal cycle studied,⁽⁷⁾ where there was a decrease in the percent of cells entering *S* (³H only) as compared to the percent of cells leaving the *S*-compartment (¹⁴C only). The fact that both flow rates are

FIG. 79. The percent of DNA synthesizing (labeled) basal cells, as determined by autoradiography, in the epidermis of hairless mice during a 24 hr period. ● points in present experiment, × mean values from previous experiment.

TABLE 38. THE DISTRIBUTION OF LABELED CELLS, BY THE TYPE OF ISOTOPE INCORPORATED, AND THE FLOW RATE OF CELLS INTO AND OUT OF DNA SYNTHESIS, USING DOUBLE LABEL AUTORADIOGRAPHY⁽⁷⁾

Observed population of cells	10 a.m. series		2 p.m. series	
	Number	t_c hr	Number	t_c hr
Labeled basal cells	1647		653	—
^{14}C only	479	0.94	257	0.56
^{14}C and ^3H	920		286	—
^3H only	248	0.49	110	0.24
Unlabeled basal cells	23805		14642	

decreasing from one period to the other is consistent with a continuous decrease in the S -indices during these periods. Differences observed in the number of cells in the categories of labeled cell types in the two series are due to the fact that in the first series the time interval between the thymidine- $2\text{-}^{14}\text{C}$ and the ^3H -thymidine was 2 hr (8 a.m. to 10 a.m.), and 3 hr in the second series of mice (11 a.m. to 2 p.m.).

Theoretical models of the flow of cells into and through DNA synthesis are shown for a steady state in Figure 80 and during changing flow rates in Figure 81. A graphic analysis is presented to facilitate

an evaluation of the changes expected in the number and the categories of labeled cell types with changing parameters, using double-label autoradiography. The relative values observed in Table 38 are consistent with the model on the assumption that the duration of DNA synthesis is constant and the flux of cells into S is changing (decreasing). Theoretical models which assume a change in the duration of DNA synthesis have been constructed, and the observed data in Table 38 are not consistent with such an assumption.

The data in Figure 79 have been derived from a 24-hr experiment using double-label autoradiography, which was designed to study flow rates throughout the diurnal cycle. Preliminary data from this experiment, during a period of increasing labeling indices, have shown ratios of the categories of labeled cell types consistent with the expected ratios, as shown in Figure 81(b) (increasing flow rates). Under the assumption of a fixed DNA-synthesis period, the relative number of cells entering synthesis should be equal to the number of cells leaving synthesis, at a time period later and equal to the duration of the

FIG. 80. Two dimensional diagram of the flux of cells into and out of the S compartment, using double-label autoradiography (abscissa, the cell cycle; ordinate, the relative number of cells in a compartment during a point in time). (a) The distribution of labeled cells in DNA synthesis during steady state when: $t_s = 6$ hr, $t_w = 2$ hr. (b) The distribution of labeled cells when: $t_s = 6$ hr, $t_w = 3$ hr.⁽⁷⁾

synthetic process. In the present experiment, preliminary calculations of two sets of flow data which were taken 7 hr apart, have shown good correlation between the cells flowing into synthesis and later flowing out of synthesis.

From all of the presently available data in the 24-hr experiment, the correlations between labeling indices and flow rates are sufficiently good to confirm our working hypothesis that, during the 24-hr period studied in the epidermis, the flux of cells from G_1 into S was actually changing, and that this change, in itself, is sufficient to cause the diurnal rhythmicity in the number of cells in DNA synthesis and in subsequent compartments of the mitotic cycle. We plan to use this technique in examining the effect on the cell cycle of the postirradiation response.

Skin Carcinogenesis from Beta Irradiation

This study was designed to test whether our observed low skin tumor incidence after beta irradiation⁽³⁾ might be enhanced by the use of a suitable promoter, as has been demonstrated for a variety of chemical carcinogens.⁽⁹⁾ Groups of 6- and 12-month-old female hairless mice and haired siblings, Hr/Anl [Anl 66], were irradiated with 2500 Rads or 5000 Rads

FIG. 82 Deposition of plutonium in bone from 1 to 360 days following subcutaneous injection of particulate ^{239}Pu Sprague Dawley female rats, castrate and noncastrate.

at a dose rate of 7.5 Rads/min, in a whole-body, beta irradiator, using a ^{90}Sr - ^{90}Y source. Two weeks after irradiation, 3 paintings per week were begun on the dorsal skin with a highly potent croton oil extract as the promoter.⁽¹⁰⁾ Both the control and painted groups, within 2 months after irradiation, showed some epilation in the 2500-Rad group and marked epilation in the 5000-Rad group. The groups have been under the painting regime for from 6 months to 1 year, and no increase in skin tumor induction has, as yet, been seen in any group. This is in marked contrast to the observations with the use of chemical carcinogens where up to 80% of the experimental animals develop tumors within 6 months of the start of painting with croton oil extracts.⁽¹⁰⁾ This result also seems to be at variance with an early report in which a lower dose of a less penetrating beta ray was used;⁽¹¹⁾ further experiments are planned to try to resolve this discrepancy.

Plutonium-Induced Osteogenic Sarcomas

Castrate Sprague-Dawley female rats injected subcutaneously with a particulate suspension of ^{239}Pu have a higher incidence of osteogenic sarcoma than normal rats with the same plutonium dose.⁽¹⁾ The present study was undertaken to establish the time pattern of distribution of plutonium in the bone, liver, and spleen, under the same conditions. Female rats were castrated at 90 days of age and injected at 130 days of age. Castrate and noncastrate Sprague-Dawley rats received similar doses of particulate ^{239}Pu , and the animals were killed at predetermined intervals for analysis. Figures 82-84 show the deposition of the radionuclide in bone, liver, and spleen from 1 week to 1 year after injection as determined by ashing analyses. The deposition patterns of castrate and normal animals are not distinguishable. The longer period of cell proliferation and bone growth in the castrate animals in the presence of similar levels of deposited ^{239}Pu , may account for the observed higher incidence of bone tumors in the castrate rats.

FIG. 81 Two dimensional diagram of the flux of cells into and out of the S -compartment, using double-label autoradiography. (a) The duration of DNA synthesis remaining constant while the flux of cells into the compartment decreases at a point in time, $t(s) = 6$ hr, $t(a) = 2$ hr. (b) The same as above, except the flux of cells into the compartment increases.⁽⁷⁾

FIG. S3 Deposition of plutonium in liver from 1 to 360 days following subcutaneous injections of particulate ^{239}Pu . Sprague Dawley female rats, castrate and noncastrate.

FIG. S4. Deposition of plutonium in spleen from 1 to 360 days following subcutaneous injections of particulate ^{239}Pu . Sprague Dawley female rats, castrate and noncastrate.

as compared to the normal rats. Studies are continuing on the long-term effects of low doses of particulate ^{239}Pu in intact and castrate rats, as well as the pattern of deposition in the femurs of the animals as determined by assay and autoradiographically.

Sarcoma Production by Subcutaneous Implants

Subcutaneous discs of Teflon, Mylar, Silastic, and Mylar-laminated sources containing $5\ \mu\text{Ci}$ of ^{90}Sr - ^{90}Y have been implanted in 5 dogs for from 36 to 42 months. No tumors, as yet, have been observed with any of these implants. Similar discs in Sprague-Dawley rats, in the case of the nonradioactive implants, have a probability of sarcoma production of from 15% to 30% per disc with a mean time to tumor production of 18 months. Radioactive discs of $5\ \mu\text{Ci}$ ^{90}Sr - ^{90}Y implanted in rats have produced tumors in 95% of the animals with a mean time to tumor production of 10 months,⁽³⁾ and all tumors were produced by 18 months. In the case of the radioactive implants, the estimated proximal beta dose rate is 300 Rads/day or about 100,000 Rads/year. In the 36 months that they have been implanted in the dogs, the sites have accumulated a dose of 300,000 Rads, and the only visible effects have been the complete epilation of skin over the implant beginning at 5

months after implantation, and the formation of fibrous scar over the source. No skin carcinomas or other local tumors have, as yet, been observed at any site. This interspecies difference is being further studied by the implantation of lower dose rate, laminated sources in additional dogs.

Tumor Induction by Nonradioactive Materials

As previously reported,⁽³⁾ all types of impermeable plastics and other materials that have been implanted subcutaneously in the Sprague-Dawley rat have produced sarcomas in the animals with tumor probabilities of from 15% to 30% per disc. In addition Millipore filters show an almost exact inverse relationship between pore size and the tumor incidence. The tumor probabilities range from 33% per disc at $0.1\text{-}\mu$ pore size to 5% at $0.45\text{-}\mu$ pore size, with only rare tumors at pore sizes over $0.45\text{-}\mu$.⁽¹²⁾ It recently has been learned that the Millipore filters used in these experiments contained the nonionic surfactant, Triton X-100 (an alkylated aryl polyether alcohol),⁽¹³⁾ which may, in some way, contribute to this effect since studies *in vitro*⁽¹³⁾ and *in vivo*⁽¹⁴⁾ have shown Triton X-100 to have marked inhibitory effects on cell growth and differentiation. Aquapel (a hydrophobic Millipore filter which repels fluids owing to a special treatment process), as well as Duralon (a nylon Millipore), Solvinert (a solvent-resistant Millipore), and Mitex (a Teflon Millipore), are being implanted in rats to study further this inverse relationship with pore size and to look for any contributing effects from surfactants on tumor incidence. The possibility that the filters selectively absorb some growth or transforming substances are also under investigation.

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RETENTION OF RADIOSTRONTIUM IN SOFT TISSUES

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PURPOSE AND METHODS

Our previous reports⁽¹⁻³⁾ have indicated that radiostrontium in most soft tissues, including testis, comes rapidly into equilibrium with blood plasma, after which the concentration ratios remain essentially constant for long periods. For these tissues, the plasma concentration curve, therefore, provides a satisfactory index of the tissue concentration integrated through time. Species and age differences in early deposition in the "exchangeable" fraction of bone and in excretory rates result in very marked differences in the plasma curve which, in turn, reflect radiation dosage levels in those organs that are not very close to mineralized tissues of the skeleton. We have made a preliminary survey of published plasma levels of radiostrontium and radiocalcium after single injections. Because questions are still being raised regarding the possible genetic effects of transmutation of radiostrontium that may be deposited in spermatozoa (presumably bound to nucleoprotein at the time of meiosis and mitosis) we have also undertaken to make measurements of ⁸⁵Sr binding in sperm. Sperm samples have been collected from dogs at intervals after injection, while plasma samples were also assayed, and the washed spermatozoa have been counted and the level of radioactivity measured.

PROGRESS REPORT

Table 39 shows representative values for a series of rat tissues which appear to reach a constant ratio of specific activity to that of plasma. These are mean values, reached after 1 to 5 hr, from animals sacrificed at intervals up to a month after injection. It was noted previously⁽³⁾ that in mice, the seminal vesicles

with contents did not reach maximum ratios to plasma until later (ratios of 2 to 3 on the second day). Tissues containing cartilage, including trachea, show increasing ratios up to several weeks, and the degree of retention in these tissues is much greater in older animals. This is also seen, to a small extent, in aorta.

In a previous report⁽²⁾ we demonstrated a marked difference between species, and with age, in the plasma retention curves. We are collecting additional data from the literature, where the measurements and conditions have been given in such a way as to make calculations possible (in most tracer studies the data are expressed in such a way that the weight of the subject must be given in order to make meaningful comparisons of tissue specific activity). These values are reduced to a common base, i.e., $\mu\text{Ci/g}$ of tissue per $\mu\text{Ci/g}$ body weight administered, and these dimensionless values are integrated through time to give a value in days from which may be derived an approximation of tissue dose resulting from a given administered amount. An acceptable integration for the first week or two can usually be obtained by graphical analysis. Some selected values are given in Table 40.

TABLE 39. RATIOS OF TISSUE: PLASMA ⁸⁵Sr AFTER SINGLE INTRAPERITONEAL INJECTION

Tissue	Ratio	Tissue	Ratio
Fat	0.25	Heart	0.56
Spleen	0.28	Lung	1.20
Liver	0.30	Small intestine	1.20
Ovary	0.37	Salivary gland	1.30
Testis	0.40	Kidney	1.40
Muscle	0.48	Skin	1.70

TABLE 40. SPECIFIC ACTIVITY $\times$ TIME FOR Ca OR Sr

Species	$\mu\text{Ci days}/\mu\text{Ci}$	Reference
Young rat ^(a)	0.15	4
Old rat ^(a)	0.59	4
300-g rat	0.80	5
Young adult rabbit	0.54	6
Rat (intratracheal)	0.61	7
4-month beagle	0.37	8
1/2-year beagle	2.9	8
Adult mongrel	1.9	6
Woman ^(a)	5.07	4
Man ^(a)	7.70	9
Human patients		Mean values
Euthyroid ^(b)	10.9	from Refer-
Thyrotoxicosis ^(b)	3.5	ences 10 and
Myxedema ^(b)	20.3	11.
Paget's disease ^(b)	2.5	
Hypoparathyroid ^(b)	11.7	
Hyperparathyroid	5.5	
Osteoporosis	9.1	

^(a) ^{45}Ca injected^(b) Including values for calcium as well as strontium.

Plasma levels are influenced, during the time when they are high, almost entirely by the rapidly exchangeable calcium pool in bone and by excretion rates. It should be possible, therefore, to use relatively simple data to make an approximation of soft-tissue dosage during the early periods after injection. A theoretical basis for this approximation has been given by Marshall.⁽¹²⁾

Where human intake of ^{90}Sr is by way of the alimentary route, as in the case of fallout, the daughter ^{90}Y is effectively not absorbed. We showed previously⁽³⁾ that in mice injected with ^{90}Sr free of ^{90}Y the translocation to testis was insignificant, although liver was found to have about four times as much ^{90}Y as ^{90}Sr . This can be compared with data of Müller,⁽¹³⁾ who injected ^{90}Y along with ^{90}Sr and found evidence of greater retention of yttrium and some accumulation in the testis.

We have attempted to measure ^{89}Sr retention in dog spermatozoa. Aberg and Gillner⁽¹⁴⁾ reported measurable values (0.1 atom of ^{89}Sr per cell, in rams given injections in the acute lethal range). Of the few sperm samples we have collected so far in adult beagles, one sample taken at two weeks showed a measurable amount. A sample of 1.5×10^8 sperm, after washing to a constant level, contained 2.8 ± 0.8 counts/min

following a total injection of 7.4×10^7 counts/min, each sperm cell containing about 2.5×10^{-16} of the total amount injected. This agrees well with Aberg's measurement⁽¹⁴⁾ on the ram.

It seems clear from preliminary estimates based on these data that the deposition of radiostrontium in sperm cannot be a significant factor in the fallout hazard. Further experiments on this and on yttrium translocation are being carried out in order to make more precise calculations.

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CELLULAR PROLIFERATION AND CARCINOGENESIS

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PURPOSE AND METHODS

This program is part of a continuing effort to describe and compare the proliferation kinetics of normal and tumor tissues. Normal tissue kinetics are measured under different functional and physiological conditions so that the effect of proliferative behavior on tumor induction can be evaluated. The kinetics of tumors are compared to the kinetics of normal tissues, including the tissue of origin, to help clarify developmental mechanisms. Finally, growth parameters of histologically similar tumors induced by different agents will be examined to evaluate the relationship of tumor cell kinetics to the inducing agent. In association with these long-term objectives, methods used in measuring proliferation kinetics and inducing tumors are being reevaluated.⁽¹⁾

PROGRESS REPORT

In earlier communications^(2,3) autoradiographic estimates of the cell cycle in the cheek pouch epithelium of adult and weanling hamsters and preliminary data from neonatal animals were presented. Additional data on neonatal animals are presented in Figure 85. The percent of mitoses labeled by 5 μ Ci of tritiated thymidine (3 HTdR) is plotted against time after I.P. injection. The transit time through DNA synthesis (T_s) estimated from the first wave of labeled mitoses is about 6 hr. In weanling and adult animals, T_s was between 9 and 10 hr. The mean transit time through the generation cycle (T_c) is estimated to be approximately 26 hr in neonatal animals, compared to about 60 hr in weanlings and 150 hr in adults. Although there appear to be changes in T_s with aging, the most significant changes occur in the postmitotic, pre-DNA-synthetic or T_{cl} period. The change in shape and subsequent disappearance of the second wave of labeled mitoses is consistent with a model in which G_1 cells randomly begin DNA synthesis at a rate based on the local demand for cell production. In the growing animal, where the demand is high, variability between cells should be reduced and a second wave reasonably well defined. In adults, where the demand for cell production is reduced and the variability increased, a second wave is difficult to demonstrate. Figure 86 is a photomicrograph of the developing cheek pouch in a day-old hamster. An epithelial anlage is formed at the junction between the skin of the cheek and the buccal

mucosa. The outer portions of this anlage are formed by basal cells with differentiation progressing toward the center. On about the seventh day a separation occurs, resulting in the formation of the pouch, Figure 87.

The results of experiments designed to look for rhythms in physiologic activity were reported last year.⁽⁴⁾ Preliminary data showed poorly defined patterns of activity and indicated that under our normal laboratory conditions (constant light) activity was nearly random throughout the day. Additional experiments have been carried out or are in progress to detect rhythms in activity, body temperature, mitotic index, and labeling index.

Temperature-sensitive, miniature radio transmitters were implanted in the abdomens of two hamsters. Temperature changes alter the transmission frequency, and changes in the orientation of the transmitters with respect to the receiving antenna produce periodic signal loss. It is possible to use the signal loss to estimate the frequency and time distribution of activity in addition to obtaining the measurements of temperature. Figure 88 illustrates a typical recording. Data obtained in this way are closely correlated with those obtained from suspension cages. At this time, it has not been possible to demonstrate systematic changes in body temperature nor to correlate temperature change with activity.

In another experiment, 24 hamsters were injected with 0.5 μ Ci g of 3 HTdR at different times during the day and killed one hour later. Six thousand randomly selected basal cells per animal were scored in cheek pouch autoradiographs as labeled or unlabeled, mitotic or interphase. The data are shown in Table 41. No significant differences have been

FIG. 85 The percent of epithelial mitoses labeled by 3 HTdR in the developing cheek pouch of newborn hamsters

FIG. 86.—Histogenesis of the cheek pouch, 1 day. $\times 100$.

found as a function of time of day. Rhythms may exist, but the level of interanimal variation is so high that extremely large samples will be required to demonstrate them. Preliminary estimates of thymi-

dine uptake, measured by mean grain counts, suggest that peak incorporation occurs in mid-afternoon. It is not clear why there are more conspicuous changes in uptake than in percent of cells labeled.

FIG. 87.—Histogenesis of the cheek pouch, 11 days after birth.

Attempts have been made to induce tumors in the cheek pouch with several different agents. In the previous report,⁽³⁾ it was observed that irradiation of a 1-cm diameter field (2,500 to 10,000 Rads) was

ineffective within the first three postirradiation months. Animals have now been observed for a total of 15 months, and the tumor incidence is zero. Because tumor induction may be affected by the size

FIG. 88.—Telemetric recording from implanted transmitter. Circular tracing indicates frequency of transmission, which is temperature dependent. Radial lines indicate periodic signal loss.

TABLE 41. MITOTIC AND LABELING INDICES IN BASAL CELLS IN HAMSTER CHEEK POUCH AT DIFFERENT TIMES OF DAY^(a)

Time.....	0600	0800	1000	1200	1400	1600	2330	0110	---
M.I. ^(b)	0.65	0.73	0.93	0.80	1.00	1.00	0.70	1.03	0.86
L.I. ^(c)	2.88	4.79	5.80	4.48	3.56	5.08	3.50	3.22	4.25

^(a)Counts are based on 6,000 randomly selected cells per animal and 3 animals per group.

^(b)M.I., mitoses per 100 cells.

^(c)L.I., labeled cells per 100 cells.

of the irradiated field, a second experiment was undertaken in which the entire pouch was irradiated (50 kVp X rays, 2,500 to 7,000 Rads). Two hundred and four animals or 408 pouches have produced no tumors within the first 6 months. Aside from early erythema and edema, the only change noted has been moderate deep fibrosis.

In addition to the previously described attempts to induce cheek pouch tumors by irradiation, 44 newborn hamsters had SV-40 inoculated into the area of the developing cheek pouch. One of these ham-

FIG. 89. Photomicrograph of SV-40 induced fibrosarcoma (Hematoxylin and eosin $\times 100$).

sters developed a lipoma at a distant site and two others subsequently developed anaplastic fibrosarcomas of the cheek pouch (Figure 89). These tumors are being carried by transplantation in the strain

of origin (Gd/Anl) and in an inbred strain (PD4/Anl). No transplants into hamsters inoculated with SV-40 at birth have been successful, suggesting that the tumors carry SV-40 antigen. Animals with second

FIG. 90.— The percent of mitoses labeled in SV-40 tumors at different times after injection of $^3\text{HTdR}$.

generation transplants were injected with $^3\text{HTdR}$ and killed at various times thereafter. Figure 90 is a preliminary plot of labeled mitoses obtained from these animals. When these data are compared to those shown in Figure 91, which are derived from connective tissue cells in newborn hamsters, it appears that T_s of normal connective tissue cells is shorter than its counterpart in the viral induced tumors.

FIG. 91. The percent of mitoses labeled in the connective tissue of the developing cheek pouch as a function of time after injection of $^3\text{HTdR}$.

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STUDIES OF RAT MAMMARY GLAND GROWTH

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PURPOSE AND METHODS

The purpose of these studies is to define growth patterns of the rat mammary gland and to explore their relationships with carcinogen sensitivity.

We report here 1) a brief description of a method to prepare broad-section autoradiographs that gives more representative histological sampling of all structural divisions of the gland, and facilitates recognition of possible regional differences in the proliferative activity and differentiation of the gland; 2) a correlation of labeling index measurements of cell proliferation obtained from autoradiographs of the gland with more easily obtained indirect parameters of gland growth.

We ask these questions: In a cycling female rat given tritiated thymidine, how do the labeling indices of the several cell subpopulations correlate with the gland DNA specific activity? Does the extent of occupancy of the fat pad by the expanding gland-epithelial structures (duct, alveolus, and end bud)

correlate with the specific activity of the mammary gland DNA? (The index of gland occupancy is approximated by the ratio of fat pad gland DNA/fat pad gland wet weight.)

Earlier, we reported the determination of specific activity of mammary gland DNA in cycling female rats given tritiated thymidine.⁽¹⁾ The specific activity of the DNA is a single index of the average proliferative activity of the entire gland epithelium. Now we report a method for preparing broad-section autoradiographs of the mammary gland which can provide information about proliferative activities of each of the several cell subpopulations over a wide area. Duct, alveolus, and end bud are not equally distributed throughout the abdominal-inguinal gland. Instead of preparing multiple biopsies whose aggregate area may be quite limited, where structures may be missed, and the orientation is awkward to reconstruct, we have devised a method to obtain broad, 4- μ -thick sections. Autoradiographs of

such broad sections contain adequate representation of all structures and preserve their anatomical relationships. The labeling index of epithelial cell subpopulations from the gland exposed to isotopes is used as a first approximation of their comparative proliferative activities.

In brief, broad-section autoradiographs of a Sprague-Dawley derived rat mammary gland are prepared from rats given 1 μ Ci/g body weight of 0.36 Ci/mM of tritiated thymidine intravenously. One hour later, the abdominal-inguinal fat pad, containing the gland proper, is excised, stretched out on glass, and fixed in cold acetone. After formaldehyde fixation, the specimen is dehydrated through graded alcohols and cleared with fat solvents. The gland, at this stage, is broad and thin with wrinkled contours. A final planar orientation of the gland in an embedding medium is required to obtain a broad section. The specimen is placed between two layers of fine monofilament polyester screen fabric held taut like two superimposed drumheads over the end of a cylindrical Teflon mold. The gland in this planar state is embedded in a methacrylate block after a method described by Norris.⁽²⁾ After polymerization, the block is removed from the Teflon mold and is machined so that the sides are perpendicular to the plane of the tissue. The specimen lies flat just beneath, and parallel to, the top surface of the block. The block is positioned in a Jung motorized sledge microtome and addressed precisely to the blade so that broad sections of the tissue can be cut. Sections, 4 cm in diameter and 4 μ thick, are mounted on 3- x 2-in slides. Autoradiographs are prepared by the dipping technique in the usual manner, and finally, the sections are stained with hematoxylin and eosin.

The labeling index of cells are found in three general zones of the section under 1000 $\times$ magnification. These are a superior lateral zone, a zone at the level of the inguinal lymph nodes, and a zone intermediate to the former zones. The end buds are concentrated in the superior lateral zones. Where possible, at least 2000 epithelial cells are counted in each zone, for each cell subpopulation, so that approximately 18,000 cells are counted per slide.

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We have correlated average breast proliferative activity data obtained by two methods. The labeling index data obtained from autoradiographs are correlated with the specific activity of mammary gland DNA obtained by a biochemical and liquid scintillation counting method. Table 42 shows the DNA specific activity for four phases of the estrus cycle

TABLE 42 THE EFFECT OF THE ESTRUS CYCLE ON THE MAMMARY GLAND DNA SPECIFIC ACTIVITY

Phase	No. of rats	Average DNA specific activity, dpm/ μ g	Range of DNA specific activity, dpm/ μ g
Proestrus	20	73	42-103
Estrus	18	117	55-186
Metestrus	19	190	91-402
Diestrus	17	62	39-116

TABLE 43 THE RELATION OF SPECIFIC ACTIVITY OF MAMMARY GLAND DNA TO THE LABELING INDEX OF MAMMARY GLAND EPITHELIAL CELLS OF ALVEOLUS, DUCT AND END BUD DURING ESTRUS AND METESTRUS

Specific activity of DNA, dpm/ μ g	Number of rats	Percentage of labeled cells							
		Alveolus		Duct		End bud			
		Estrus	Met-estrus	Estrus	Met-estrus	Estrus	Met-estrus		
50-75	3			0.63	-	0.71	-	13.29	-
76-125	3	5	3.37	4.36	4.56	5.51	13.33	13.34	
126-175	6	4	4.92	4.89	5.67	6.93	15.84	13.54	
176-225	2	2	5.86	7.24	7.39	7.67	17.96	18.77	
226-425	1	1		15.37	-	7.06		16.00	

in 60-day-old rats given tritiated thymidine. Diestrus and proestrus are lowest, metestrus is highest and estrus is intermediate. Note the wide range of variation, particularly for estrus and metestrus, and how the specific activity values for these two phases have a large region of overlap. In Table 43 are the results for the 14 estrus and the 15 metestrus rats chosen for initial analysis because their DNA specific activity values are distributed over the complete range.

Rats in both estrus and metestrus phases were grouped by the specific activity levels indicated. The average labeling index for duct, alveolus, and end bud was calculated for each animal by averaging data collected from three anatomical zones of the gland. The labeling indices recorded are the average of individual rats of the same cycle phase and specific activity level. There is a correlation of ascending DNA specific activity values and the rising labeling indices of alveolus, duct, and end bud. In those levels of DNA specific activity in which estrus and metestrus overlap, 75 to 125, 126 to 175, and 176 to 225 dpm/ μ g, duct labeling index values are slightly higher than alveolar values. Estrus and metestrus values are quite similar for both of these cell subpopulations. Only metestrus rats are found in the highest specific activity level, (226 to 425 dpm/ μ g). Also of interest

TABLE 44. CORRELATION OF MAMMARY GLAND GROWTH RATE AND RELATIVE SIZE OF THE GLAND TO THE FAT PAD IN WHICH IT RESIDES, EXPRESSED BY THE MAMMARY GLAND SPECIFIC ACTIVITY VS. THE TOTAL GLAND FAT DNA/WET WEIGHT OF THE GLAND FAT PAD

Specific activity of DNA, dpm/ μ g	Number of rats		Gland fat pad DNA/gland fat pad wet weight μ g/mg	
	Estrus	Metestrus	Estrus	Metestrus
50-75	3		1.295	-
76-125	3	5	1.435	1.718
126-175	6	4	1.205	1.354
176-225	2	2	0.989	1.159
226-445		4		0.857

in this group is a sharp rise in the alveolar labeling while the duct value drops slightly. In contrast, at the low end of the specific activity range, only estrus rats are represented and the labeling index of both duct and alveolus are very low. Labeling indices of end bud corresponding to the lowest level of DNA specific activity are relatively high and show only an average increase up to 40% as the specific activity level rises more than threefold. For the end bud, also, the average labeling index is similar for both estrus and metestrus at any given DNA specific activity level.

An index of gland occupancy of the fat pad is approximated by the ratio of fat pad gland DNA/fat pad gland wet weight. In this fat pad gland, a single fat cell is much larger than a single epithelial cell. Changes in the number of the epithelial cells sharply affect the total DNA but do not proportionately affect the wet weight of the gland fat pad. In Table 44, the ratios of fat pad gland DNA fat pad gland wet weight are correlated with the ascending levels of mammary gland DNA specific activity

for estrus and metestrus. For the higher specific activity levels (higher gland growth rate) the values of the ratio are lower. That is, the fat pad is currently less occupied by the epithelial structures of the gland, which are now growing more rapidly. For the lower specific activity values the ratio is higher, indicating the fat pad is relatively more occupied by the gland epithelial structures which are currently growing at a slower rate. This relationship of gland occupancy to growth rate warrants further study.

CONCLUSIONS

1. A method has been devised to prepare autoradiographs of broad areas of rat mammary gland suitable for study of cell kinetics of all three structural divisions: duct, alveolus, and end bud. The anatomical interrelationships of these structures in a given broad plane is preserved, and comparisons of large areas are possible.

2. In 60-day-old female rats, the average incorporation of tritiated thymidine in mammary gland DNA varies with the phase of the estrus cycle and is reflected in specific activity of DNA and labeling index. Estrus and metestrus labeling indices of duct, alveolus, and end bud can be correlated with DNA specific activity levels.

3. The relative size of the mammary gland to the fat pad in which it resides can be correlated with the average growth rate of the gland in estrus and metestrus.

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AN ATTEMPT TO GROW HeLa CELLS IN AN EISLER-WEBB NEPHELOSTAT

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PURPOSE AND METHODS

The Nephelostat is a photocell-controlled continuous-culture device for the automatic maintenance of bacteria in balanced growth.⁽¹⁾ It should be possible to adapt this device for the continuous culture of mammalian cells.

HeLa cells growing in suspension were obtained

from McArdle Laboratory at the University of Wisconsin and were placed in a slightly modified Nephelostat growth tube. Culture medium was added and removed with 1-mm diameter Teflon tubing connected to a peristaltic pump that had silicone rubber pumping tubes. Cell growth was recorded automatically by the Nephelostat and by counting samples in the Coulter counter.

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The cells grew quite well in this system for approximately seven weeks, at which time the system became contaminated with mold. This experiment showed, therefore, that the procedure is feasible.

Because the standard Nephelostat growth tube has a volume of only 25 ml, subsequent experiments have involved attempts to enlarge the capacity of the system to 500 ml so that sufficient cells can be obtained for biochemical studies. Two major difficulties have been encountered in these experiments.

First, larger diameter tubing was needed to supply medium to the larger growth reservoir. Latex rubber tubing allegedly suitable for intravenous feeding was used and was found to contain substances which were extracted into the medium and which were highly toxic to the cells. This problem was overcome through the use of medical grade silicone rubber tubing, which was nontoxic to the cells.

Second, cells are normally maintained in a single cell suspension in the Nephelostat by bubbling a mixture of air and 5% CO₂ through the reservoir. With the larger reservoir, increased bubbling was required, causing an unacceptable amount of foaming. An attempt was made to suppress the foaming by adding a silicone antifoam preparation. Prior addition of the antifoam to Petri-dish cultures of HeLa cell monolayers had resulted in no visible effects on cell morphology and rate of growth. When the antifoam was added to the suspension culture, however, it

mixed more extensively with the medium because of the agitation provided by the bubbling. The cells then ingested the antifoam droplets (which appeared as cytoplasmic granules), and consequently failed to grow. When these cells were removed from the Nephelostat and returned to Petri-dish cultures they began to regurgitate the antifoam. This was indicated by the steady decrease in the granularity of the cells and by the appearance of antifoam droplets in the medium. This process continued for several days, through several changes of medium, after which the cells reacquired their normal appearance and resumed a normal rate of growth. It is clear, therefore, that the antifoam was not chemically toxic to the cells but rather interfered with their function through a process of physical engorgement. In any case, antifoam and, consequently, agitation by bubbling, cannot be used. Future experiments will attempt to determine whether stirring can be used to keep the cells in suspension.

CONCLUSION

HeLa cells can be grown in continuous culture in the Nephelostat, but further study is required to determine the best conditions for maintaining the cells in an enlarged Nephelostat growth chamber.

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ENZYME REGULATION IN RAT LIVER: FUNCTIONAL PROPERTIES OF THE ORNITHINE AMINOTRANSFERASE MOLECULE

Carl Peraino

PURPOSE AND METHODS

Ornithine aminotransferase, an amino acid catabolizing enzyme, is located in rat liver mitochondria.⁽¹⁾ It is strongly regulated *in vivo* both by feedback inhibition and by dietary and hormonal alteration of its turnover.⁽²⁻⁵⁾ These characteristics render ornithine aminotransferase a useful tool for studies of the mechanism of enzyme regulation. The enzyme was purified, and several of its chemical, physical, and structural properties were studied.⁽⁶⁾ This report describes an extension of this investigation and includes an examination of functional properties of the enzyme under a variety of experimental conditions. This study is still preliminary because much time is required to perfect or develop techniques. However the results obtained are encouraging.

The ornithine aminotransferase reaction was examined in two half-reactions. Each half-reaction is investigated under a variety of conditions. The overall ornithine aminotransferase reaction is as follows:

First half-reaction

ornithine + ornithine aminotransferase-pyridoxal phosphate → pyrroline carboxylate + ornithine aminotransferase-pyridoxamine phosphate

Second half-reaction

alpha ketoglutarate + ornithine aminotransferase-pyridoxamine phosphate → glutamate + ornithine aminotransferase-pyridoxal phosphate

The first half-reaction is studied by reacting ¹⁴C-

ornithine with the enzyme, removing the enzyme, and resolving the resultant mixture of ornithine and pyrroline carboxylate on a Dowex 50 cation exchange column. The second half-reaction is studied by treating the enzyme with ornithine, removing the ornithine and pyrroline carboxylate by Sephadex treatment, and reacting the pyridoxamine form of the enzyme with ^{14}C - α -ketoglutarate. The mixture of α -ketoglutarate and glutamate obtained are again resolved by ion exchange chromatography.

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Figure 92 shows the stoichiometry of each half-reaction. These results indicate that one mole of enzyme (assuming a molecular weight of 132,000—see Reference 9) reacts with two moles of ornithine. This relationship is not strictly observed in the second half-reaction, in which one mole of enzyme reacts with 1.5 moles of α -ketoglutarate. The reason for this lower value is not known at present, but it may represent a partial loss in the functional capacity of the enzyme after its conversion to the pyridoxamine form or it may indicate a change in the molecular weight of the enzyme during the reaction.

Figure 93 indicates activity vs. pH for the complete enzyme reaction. The curve is symmetrical, with an optimum at approximately pH 8.2.

FIG. 92.—Stoichiometry of the ornithine amino transferase half-reactions.

Fig. 93.—Effects of pH on the complete ornithine aminotransferase reaction.

FIG. 94.—Effects of pH on the ornithine aminotransferase half-reactions.

Figure 94 shows the effects of pH on each half reaction. The responses of the two half-reactions to pH changes differ markedly, both from each other and from the response of the complete reaction. Combining these results for the half-reactions (Figure 94) and proceeding from low to high pH reveals that the ascending limb of the pH curve for the whole reaction (Figure 93) corresponds to that of the first half-reaction (Figure 94), and the descending limb for the whole reaction (Figure 93) corresponds to that of the second half-reaction (Figure 94). The composite pH curve obtained when the curves from the two half-reactions (Figure 94) are combined, however, is displaced to the right (higher pH) of the pH curve for the whole reaction (Figure 93). The significance of this difference is under investigation.

CONCLUSION

A procedure was devised to study the two half-reactions of ornithine aminotransferase. The results obtained indicate that: 1) Two molecules of each substrate react with one molecule of enzyme. 2) The responses of each half-reaction to changes in pH differ from each other and from that of the complete reaction. 3) The pH curve for the complete reaction apparently is a composite of the curves for the half-reactions. The general approach described in these experiments appears to be well-suited to further intensive investigation of the functional properties of the ornithine aminotransferase molecule.

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METABOLIC AND MORPHOLOGIC STUDIES OF HEPATOCARCINOGENESIS IN THE RAT

Carl Peraino, R. J. Michael Fry, and Everett Staffeldt

PURPOSE AND METHODS

In previous studies we demonstrated that the production of hepatomas in rats by the feeding of acetylaminofluorene (AAF) was substantially decreased if phenobarbital was also included in the diet.⁽¹⁾ This protective effect of phenobarbital probably resulted from a stimulation of detoxifying systems in the liver.⁽²⁾ In a subsequent study⁽³⁾ an investigation was made of the effects of varying the time of exposure to a given level of dietary AAF on the production of hepatic tumors. In this experiment the effects of phenobarbital were also studied, but the phenobarbital was fed *after* the rats had been exposed to AAF rather than simultaneously with the AAF as in the previous experiment.⁽¹⁾ The survival of rats fed AAF followed by phenobarbital was less than that of rats fed AAF followed by a diet not containing phenobarbital.⁽³⁾ This suggests that the damage produced by AAF was amplified by the subsequent administration of phenobarbital. Now we report further results of the experiment involving the sequential feeding of AAF and phenobarbital.⁽³⁾

While the end point in the previous experiment was the survival time of the rats, the end point in the present experiment was the incidence of hepato-

mas. In order to obtain these data, at least 3 rats from each group were killed at intervals throughout the experiment. These intervals were 56, 84, 98, 113, 127, 161, 190, 232, and 282 days after the beginning of the experiment. At autopsy, the animals were examined for tumors and other lesions. In the majority of cases where hepatomas were obvious or where careful examination of all lobes revealed no abnormality, no tissues were taken for histological examination. Where an abnormal appearance was found, and in a number of apparently normal or tumorous livers, tissues were taken for preparation of sections. The incidence of hepatomas was compiled from the pooled data from macro- and microscopic examination. Other details of the experimental procedure have been described previously.^(1,3) In the experiments involving maintenance of rats on diets containing AAF and AAF plus phenobarbital, the proliferative activity of cells in the liver has been investigated. ³HTdR, 0.5 μ Ci/g, 0.36 Ci/mM, was injected intraperitoneally and the rats were sacrificed 1 hr later. The labeling index of hepatocytes and littoral cells was determined for the 6 treatment groups shown in Table 45.

In order to study whether phenobarbital had an

TABLE 45. INCIDENCE OF HEPATOMAS IN RATS MAINTAINED ON PHENOBARBITAL FOLLOWING A PERIOD OF FEEDING AAF (0.02%)

Treatment	Time on AAF, weeks	No. of rats	No. of rats with hepatomas
AAF	2	26	2
AAF followed by phenobarbital	2	26	8
AAF	4	26	18
AAF followed by phenobarbital	4	26	25
AAF	6	26	23
AAF followed by phenobarbital	6	26	26

FIG. 95. The incidence of hepatomas in rats treated with AAF for 2, 4, and 6 weeks and then given a diet containing phenobarbital. Dashed line, AAF followed by phenobarbital; solid line, AAF.

early, and perhaps temporary effect on proliferation of hepatocytes, 6-week-old male SD/Anl [Anl 66] rats were maintained for 3 days on control diet plus 0.5% phenobarbital and injected with $^3\text{HTdR}$ and sacrificed 1 hr later. In order to establish the labeling index of both binucleate and mononucleate hepatocytes, smears were prepared. The livers were perfused with calcium and glucose-free Hank's solution containing collagenase and hyaluronidase, sliced, agitated in a water bath, sieved, and spread on slides. Autoradiographs were prepared in the standard way, and the labeling index was determined on samples of 1000 to 2000 hepatocytes.

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The effect of the AAF in the diet for 2, 4, and 6 weeks, followed by a return to control diet with

and without phenobarbital added, is shown in Table 45 and Figure 95. The incidence of hepatomas is higher in the rats maintained on the diet containing phenobarbital in all the groups, though the difference is very small in the 6-week group owing to the high incidence of hepatomas, irrespective of the post-AAF treatment. An analysis of variance showed that the incidence of hepatomas was different in the two treatment groups at the 5% level.

It was also found that the hepatomas occurred somewhat earlier in the rats maintained on control diet plus phenobarbital subsequent to a 2-week AAF treatment. In the group of rats receiving only AAF for 2 weeks, only two tumors were found; the first occurred at 190 days and the other at 282 days. In the counterpart group given phenobarbital tumors were found on day 113, and five rats had tumors by 282 days. It is now clear that the design of the experiment is not the most suitable to show the significance of the difference in the two treatments.

The results of the autoradiographic experiments on CRL:CD(SC) rats are shown in Table 46. Because these rats are only 6 weeks old, the labeling indices are high. The labeling indices of the hepatocytes and littoral cells of the AAF (0.01%) plus phenobarbital (0.05%) were significantly different from the control value at the 2% and 5% levels respectively. It is likely that these determinations will be more informative in the two older age groups now under study. The above experiment also did not eliminate the possibility that the addition of phenobarbital to the diet might result in an early and temporary effect on proliferative activity. This question and whether there was any difference in the labeling index of mononucleate and binucleate hepatocytes was investigated. It was important to establish whether phenobarbital did increase DNA synthetic activity, as has been reported, but not documented.⁽⁴⁾

TABLE 46. THE PROLIFERATIVE ACTIVITY OF CELLS IN LIVER OF CRL:CD(SC) RATS ON VARIOUS DIETS FOR TWO WEEKS

Treatment	Labeling index, mean $\pm$ S.D.	
	Hepatocytes	Littoral cells
1. Control diet	2.8 $\pm$ 0.6	5.8 $\pm$ 0.8
2. + Phenobarbital 0.05%	3.2 $\pm$ 1.7	6.9 $\pm$ 5.0
3. + AAF 0.01%	2.1 $\pm$ 1.9	5.0 $\pm$ 2.9
4. + AAF 0.02%	3.5 $\pm$ 0.6	6.6 $\pm$ 0.2
5. + AAF 0.01% + Phenobarbital	4.3 $\pm$ 0.1	8.0 $\pm$ 0.8
6. + AAF 0.02% + Phenobarbital	2.6 $\pm$ 1.0	6.8 $\pm$ 0.1

because of importance in the possible relationship of DNA synthetic activity and the neoplastic transformation.

In the SD Anl[Anl 66] rats, on the control diet, the labeling index of the hepatocytes was 0.36 ± 0.26 ; it was 1.45 ± 0.53 for those given 0.05% phenobarbital in the diet for 3 days. These values are significantly different at the 5% level. The percentage of binucleate hepatocytes was 23.8 ± 0.5 and the labeling index for binucleate parenchymal cells was not greater than for mononucleate cells. There appears to be a surprisingly large strain difference in the labeling index of hepatocytes, namely, 0.36 ± 0.3 in the SD/Anl[Anl 66] rats and 2.8 ± 0.6 in the CRL:CD(SD) rats, although the ages of the rats and the diet were the same in both groups.

CONCLUSION

The indication that phenobarbital influences the carcinogenetic effect of AAF when given after the

treatment with carcinogen must be substantiated. If the finding is confirmed it will open up possibilities for investigating the biochemical events associated with neoplastic transformation.

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IMMUNOFLUORESCENT LOCALIZATION OF ORNITHINE AMINOTRANSFERASE IN RAT LIVER

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PURPOSE AND METHODS

Ornithine aminotransferase (L-ornithine:2-oxoacid aminotransferase) can be used to study the nature of enzyme regulation because it is regulated *in vivo* by feedback inhibition⁽¹⁾ and by dietary and hormonal regulation of its turnover.⁽²⁻⁵⁾ Activity also varies among different transplantable hepatomas^(7,8) and among different classes of mitochondria.⁽⁹⁾ For these reasons, it was of interest to determine whether the distribution of the enzyme could be studied by histochemical methods. This report describes the localization of ornithine aminotransferase in rat liver by an indirect fluorescent antibody technique.

Crystalline ornithine aminotransferase from rat liver⁽¹⁰⁾ was emulsified with an equal volume of Freund's complete adjuvant and injected into rabbits for antibody production. Another group of rabbits was injected with Freund's adjuvant alone to produce a control serum. The specificity of the antiserum for ornithine aminotransferase was verified by Ouchterlony double diffusion experiments and immuno-electrophoresis, using both crude tissue extracts and pure enzyme as sources of antigen. Single precipitin bands were observed in all cases.

Male 5-week-old SD Anl[Anl 66] rats were fed either a pelleted diet containing 0% protein or 60% protein ad libitum. In addition some rats were fed a standard laboratory diet containing 24% protein. The effects of these diets on the level of hepatic ornithine aminotransferase in rats has been reported.^(5,6) The 60% protein diet induces high levels of this enzyme, whereas rats fed the 0% diet have little or no hepatic ornithine aminotransferase. Livers of rats fed the standard laboratory diet have about one-fifth as much ornithine aminotransferase activity as those fed the 60% protein diet.

After 4 days on these two diets the rats were killed and frozen sections of liver were prepared and fixed in methanol. In one experiment, pellets of mitochondria from livers of rats fed the 60% diet and the 0% diet were prepared by differential centrifugation and sectioned. The fixed sections were stained by the indirect fluorescent antibody procedure previously described,⁽¹¹⁾ except that Eriochrome black was used as the counterstain. The slides were

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FIG. 96 Section of pelleted liver mitochondria from a rat fed the 60% protein diet. The section was treated with ornithine aminotransferase antiserum and then with fluorescein labeled antirabbit goat globulin.

examined with a Leitz Ortholux microscope equipped for fluorescent microscopy.

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Brilliant fluorescence (Figure 96) was observed in sections of pelleted rat liver mitochondria from rats fed the 60% protein diet treated with anti-serum and then with fluorescein labeled antirabbit goat globulin. No specific fluorescence was seen in similar sections treated with control rabbit serum. Sections of rat liver mitochondria from rats fed the 0% protein diet and treated either with anti-serum or with control serum did not fluoresce.

The ornithine aminotransferase specific fluorescence in livers from rats fed the 60% protein diet was diffusely distributed throughout the cytoplasm, but not in the nuclei of hepatic cells (Figure 97). Fluorescence was absent in sections from the same liver

FIG. 97 Ornithine aminotransferase immunofluorescence in rat liver section from rat fed the 60% protein diet. The section was treated with antiserum and fluorescein labeled antirabbit globulin.

treated with control serum. When specifically stained sections from rats fed the 60% protein diet were examined with low power, patches of fluorescent cells appeared to fill the entire lobule. In contrast, fluorescent cells in sections of livers of rats fed the standard diet containing 24% protein were less numerous and more randomly distributed throughout the lobule. The livers of rats fed the 0% protein diet and treated with anti-serum showed no specific fluorescence.

CONCLUSION

The fluorescent antibody results reported here correlate well with measurements of ornithine aminotransferase activity in extracts of livers from rats fed similar diets.^(5,6) The technique should prove useful in further studies of the localization of ornithine aminotransferase in normal and diseased livers.

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OPTIMUM BEAM ENERGY AND GEOMETRY FOR NEUTRON CAPTURE THERAPY

Norman A. Frigorio

PURPOSE AND METHODS

In a recent diffusion treatment of the penetration of neutrons into tissue, Ryabukhin⁽¹⁾ concluded that the optimum neutron energy depended only on tumor depth, rising from 100 eV for a tumor at the surface to 2.5 MeV at 14 cm. Unfortunately, this treatment ignores the neutron-gamma dose to the patient from the beam itself. This effect is very marked and, unless carefully controlled, would lead to demise of the patient from radiation at a beam level still too low to significantly affect the tumor.⁽²⁾

We have continued our previous examination of this question,⁽²⁾ using both theoretical and experimental methods. For theoretical computations program BIM-130 was used⁽²⁾ and dose-space-energy distributions computed for a cubical phantom 16.35 cm on each side at various neutron energies and for various beam areas. Beams were taken to be plane collimated and normal to the $X = 0$ plane of the phantom. Composition and density were those of tissue equivalent liquid,⁽³⁾ and temperature was taken as 293°K.

The experimental phantom was of the same size, composition, temperature, and orientation as above, except that it was contained in a tank of 0.03 cm stainless steel type SS-304. The beams used were the 2 keV Sc filtered beam, and the 25 keV Fe filtered

beam, of the Idaho Divisions Materials Testing Reactor (MTR). Spectrometry of these beams was accomplished with proton recoil and ³He spectrometers. The 2 keV beam proved to be contaminated with 30% or more of neutrons over 100 keV, so that no further examination was attempted. The 25 keV beam contained less than 1% of neutrons over 40 keV, so that it was used without modification for depth dose studies. Measurements were made with tissue equivalent chambers, BF₃, ³He, and ²³⁵U counters as in earlier work. For some measurements, a model human head was used* with organs and tissues filled with tissue-equivalent gels,⁽⁴⁾ skull and other bones filled with an aqueous slurry of Ca₃(PO₄)₂, made into a gel by addition of agar.

PROGRESS REPORT

As our previous computations had indicated that optimum energies would lie around a few keV and optimum source areas around 50 cm², this region was explored in greater detail. Results are presented in Table 47 for the tumor-to-skin ratio (TSR) at 6.5 cm. This is the ratio of the total dose to a tumor located at a depth of 6.5 cm and containing 3.5×10^{-3} moles kg⁻¹ of ¹⁰B to the total dose near the skin. TSR taken in this way yields a "worst case"

* The Visible Head, Renewal Products, Inc., Mineola, N. Y.

TABLE 47. TUMOR-TO-SKIN RATIO AS A FUNCTION OF BEAM SIZE AND NEUTRON ENERGY

Beam energy eV	Tumor-to-skin ratio ^(a)	
	10 × 10 cm beam	1 × 1 cm beam
10	1.38	0.92
100	1.70	1.41
1,000	2.62	1.71
5,000	2.26	1.29
25,000	1.17	0.41
50,000	0.88	0.25
1,000	3.11 ^(b)	
5,000	2.00 ^(b)	
25,000	0.92 ^(b)	

^(a) Tumor taken as 1 cm³ in volume at a depth of 6.5 cm and containing 3.5×10^{-2} moles ¹⁰B/kg tumor. Skin taken as a 1 cm³ cube of normal tissue, free of ¹⁰B, located at a depth of 0.5 cm. Beam plane collimated, square in cross section, entrant perpendicular to the X = 0 face of a cubical phantom of equivalent liquid 16.35 cm on each side.

^(b) TSR here taken with skin reference an infinitesimal volume at X = 0. Beam at 25 keV was a circle of area 15.5 cm².

figure of merit for brain tumors, as 6.5 cm depth in the phantom used is equivalent to the center of a human brain.

From Table 47, it is clear that the larger beam invariably gives a higher TSR than the smaller, although the difference decreases somewhat with decreasing energy. Computations were also made for beams of intermediate sizes but, as TSR increased monotonically with beam area, only the extremes are shown here. Because 10 × 10 cm is about the area of the front of a human head, a much larger beam is impracticable.

It is also clear from Table 47 that the optimum energy is about 1 keV. Unfortunately, a reasonable dose rate for therapy, about 100 Rads min⁻¹, would require a flux of about 10^{10} n cm⁻² sec⁻¹, and no 1 keV source is known that even approaches this figure. Some hope may be held for a 2 keV beam via Se filtration of a reactor spectrum. The 2 keV beam available at present (see above) has a flux of only 2×10^6 n cm⁻² sec⁻¹ and a completely unacceptable contamination by high energy neutrons. The possibility exists, however, that these objections can be overcome by appropriate engineering maneuvers. The available 25 keV beam, while adequately monoenergetic, has an available flux of only 2.2×10^5 n cm⁻² sec⁻¹, although this, too, may be amenable to engineering improvement. In either case, reengineering can hardly take the form of a 10^4 increase in reactor power since the present power at the 2 and 25 keV facilities is already 40 MW. Rather, the output spectrum must be modified to increase

the fraction in the 2 to 25 keV region. Thus, an intermediate energy reactor must be designed, or found, with an adequate neutron output in the 2 to 25 keV region. We examined this question at the epithermal neutron facility of the Medical Research Reactor (MRR) at Brookhaven National Laboratory, using foil, ³He, ⁶Li, and proton recoil spectrometry. As predicted theoretically, the flux in the 25 keV region was 3.2×10^7 n cm⁻² sec⁻¹ per MW, requiring a somewhat unrealistic 300 MW in reactor power for an output flux of 10^{10} n cm⁻² sec⁻¹. This does represent an improvement over the 10,000 MW required at the more highly thermalized MTR facility, however, suggesting that a facility can be designed or found which is sufficiently high in epithermal neutrons to provide the required flux at some reasonable reactor power.

The patterns of tumor dose, gamma dose, and total dose at 1, 5 and 25 keV are shown in Figures 98-100. As neutron energy increases above 1 keV, the proton recoil dose near the surface increases rapidly, while the tumor and gamma doses slowly decrease. This results in a monotonically decreasing TSR above

FIG. 98. Depth dose distributions in head phantom irradiated by a 10 × 10 cm beam as computed with BIM-130. D_B = dose to tumor containing 3.5×10^{-2} moles kg⁻¹ ¹⁰B; D_T = gamma dose to normal tissue; D_G = total dose to normal tissue, 1 keV neutron energy.

FIG. 99. —As for Figure 98, 5 keV neutron energy

1 keV. Below 100 eV, although not shown, the gamma and total doses remain nearly constant, but the tumor dose begins a fairly rapid drop, especially at penetrations greater than 3 cm, depressing TSR values. Between 100 eV and 1 keV a fairly broad optimum region exists wherein a choice of energy would be dictated by the precise shape, size, ^{10}B concentration and position of the tumor. Figure 100 indicates the excellent agreement to be obtained in comparisons between experiment and BIM-130 computations.

Included in Table 47 are the 6.5 cm TSR values referred to the epidermis, i.e., at $x = 0$. As skin is a critical tissue in radiotherapy, these TSR values are somewhat more relevant to actual therapy. For reference the 12 cm TSR values from Figures 98, 99, and 100 are 0.43, 0.32, and 0.14, respectively. Thus, 1 keV is the better energy, even at depths equivalent to a tumor in the center of a human torso.

Theoretical studies will continue to explore the relationship of beam energy, beam geometry, tumor geometry, and composition until we are able to de-

FIG. 100. As for Figure 98, 25 keV beam of circular cross section, 45.5 cm^2 in area. D_N = neutron dose to normal tissues; D_G = gamma dose to normal tissue. D_B as in Figure 98. Experimental points (circles) are compared with those computed via BIM 130 (triangles).

fine optimum conditions for all feasible tumors in head and body for different-sized humans, from infants to adults. These, in turn, will guide our detailed engineering design of therapeutically useful neutron facilities. In any case, the information now available makes a strong case for intermediate spectrum reactors as a type of facility, regardless of the eventual details of construction and operation, so that feasibility studies of such reactors are under way.

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DEPTH DOSE COMPUTATIONS IN SLAB, CYLINDRICAL AND ANTHROPOMORPHOUS PHANTOMS

Norman A. Frigerio

PURPOSE AND METHODS

Computations of the neutron dose and LET distributions have been published for a semi-infinite slab 30 cm thick and for a right circular cylinder 30 cm in diameter and 60 cm high.^(1, 2) The differences between these two were quite large, so that the question arose as to whether even the cylinder provided an adequate phantom for Standard Man. We have attacked this problem with our BIM-130 program,⁽³⁾ examining the dose and LET distributions in finite slabs and in an eleven-region anthropomorphic phantom⁽⁴⁾ under nongeometric computational conditions as near as feasible to those used previously.

PROGRESS REPORT

Computations were performed at a number of representative neutron energies incident on the phantoms as plane collimated beams parallel to the x axis. The slab phantom was 24 cm in x , 32 cm in y , and 160 cm in z , with composition 6.216 in H, 0.1789 in N, 2.898 in O,

0.626 in C, 0.0093 in Na, and 0.0093 in Cl, all given in atoms $\text{cm}^{-3} \times 10^{22}$. The Standard Man, described elsewhere in this report,⁽⁴⁾ was filled with standard soft tissue of H, N, O, C⁽²⁾ in all eleven regions, at unit density. Temperature was taken as 293°K throughout. At least 2×10^5 interactions were recorded at each energy, so that each energy run required about 300 min on the IBM-360/75.

CONCLUSION

Results are shown in Tables 48 and 49 in comparison with semi-infinite slab and right circular cylinder results published previously. It is evident that significant differences exist even between the right circular cylinder and the anthropomorphic Standard Man. Thus, not only is the cylinder inadequate, but even the anthropomorphic phantom may not be adequate. We will test this proposition in future work, using geometric phantoms yet more closely resembling the Standard Man until a representation is obtained whose results are essentially unaffected by further refinement.

TABLE 48. COMPUTED NEUTRON FLUX TO NEUTRON DOSE FACTORS FOR SEVERAL PHANTOMS AND ENERGIES, TAKEN ALONG THE MIDLINE OF THE PHANTOM

Phantom ^(a)	Semi-infinite slab, #1	Finite slab, #2	Right circular cylinder, #3	Right circular cylinder #4	Standard Man, #5
Program.....	05R	BIM 130	05R	BIM 130	BIM 130
Size (x, y, z) cm.....	(30, ∞ , ∞)	(24, 30, 160)	(30, 30, 60)	(30, 30, 60)	—
2.5 MeV, 0 cm	4.1×10^{-9}	3.69×10^{-9}	3.7×10^{-9}	3.67×10^{-9}	4.22×10^{-9}
6 cm	2.4×10^{-9}	2.58×10^{-9}	2.0×10^{-9}	3.02×10^{-9}	2.72×10^{-9}
12 cm	1.1×10^{-9}	1.17×10^{-9}	0.95×10^{-9}	1.52×10^{-9}	1.36×10^{-9}
18 cm	0.45×10^{-9}	0.534×10^{-9}	0.50×10^{-9}	0.67×10^{-9}	0.622×10^{-9}
24 cm	0.19×10^{-9}	0.181×10^{-9}	0.22×10^{-9}	0.27×10^{-9}	—
30 cm	0.065×10^{-9}	—	0.04×10^{-9}	0.086×10^{-9}	—
0.5 MeV, 0 cm	1.9×10^{-9}	1.92×10^{-9}	1.4×10^{-9}	1.64×10^{-9}	2.19×10^{-9}
6 cm	3.8×10^{-10}	3.84×10^{-10}	1.1×10^{-10}	5.93×10^{-10}	7.75×10^{-10}
12 cm	4.2×10^{-11}	5.01×10^{-11}	6.0×10^{-11}	9.15×10^{-11}	1.19×10^{-10}
18 cm	4.2×10^{-12}	6.19×10^{-12}	8.0×10^{-12}	1.41×10^{-11}	3.43×10^{-12}
24 cm	—	7.64×10^{-13}	8.0×10^{-13}	2.26×10^{-12}	—
30 cm	—	—	1.0×10^{-13}	3.50×10^{-13}	—

^(a) Standard Man described elsewhere,⁽⁴⁾ as are the parameters for computation of phantom #1⁽¹⁾ and #3⁽²⁾. Phantoms 1 and 2 contained H, N, O, C in Standard Man proportions,⁽¹⁾ 3, 1, and 5 in standard muscle proportions.⁽²⁾ Phantoms were irradiated with infinite area, plane collimated beams parallel to the x axis. Cylinders had axes parallel to the z axis. Factors are given in $\text{Rad} \cdot \text{n}^{-1} \cdot \text{cm}^2$.

TABLE 49. COMPUTED NEUTRON FLUX TO GAMMA DOSE FACTORS FOR SEVERAL PHANTOMS AND ENERGIES, TAKEN ALONG THE MIDLINE OF THE PHANTOM

Phantom ^(a)	Semi-infinite slab, 1	Finite slab, 2	Right circular cylinder, 3	Right circular cylinder, 4	Standard Man, 5
Program	05R	BIM 130	05R	BIM 130	BIM 130
Size (x,y,z) cm.....	(30, ∞, ∞)	(24, 30, 160)	(30, 30, 60)	(30, 30, 60)	—
2.5 MeV, 0 cm	2.7×10^{-10}	1.99×10^{-10}	1.2×10^{-10}	1.09×10^{-10}	1.37×10^{-10}
6 cm	5.5×10^{-10}	3.56×10^{-10}	3.0×10^{-10}	3.11×10^{-10}	3.00×10^{-10}
12 cm	5.1×10^{-10}	3.56×10^{-10}	3.8×10^{-10}	3.55×10^{-10}	2.94×10^{-10}
18 cm	4.0×10^{-10}	2.64×10^{-10}	3.0×10^{-10}	2.58×10^{-10}	1.56×10^{-10}
24 cm	2.6×10^{-10}	1.66×10^{-10}	1.3×10^{-10}	1.42×10^{-10}	—
30 cm	1.8×10^{-10}	—	0.20×10^{-10}	0.54×10^{-10}	—
0.5 MeV, 0 cm	3.1×10^{-10}	2.15×10^{-10}	2.0×10^{-10}	1.91×10^{-10}	1.97×10^{-10}
6 cm	5.3×10^{-10}	4.48×10^{-10}	4.7×10^{-10}	3.67×10^{-10}	2.41×10^{-10}
12 cm	4.0×10^{-10}	2.97×10^{-10}	3.0×10^{-10}	2.96×10^{-10}	2.63×10^{-10}
18 cm	2.5×10^{-10}	1.67×10^{-10}	1.4×10^{-10}	1.75×10^{-10}	1.72×10^{-10}
24 cm	1.6×10^{-10}	0.645×10^{-10}	0.48×10^{-10}	0.866×10^{-10}	—
30 cm	0.83×10^{-10}	—	0.15×10^{-10}	0.338×10^{-10}	—

(a) Conditions as for Table 48.

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INSTRUMENTS AND MATERIALS FOR STUDIES OF DEPTH DOSE DISTRIBUTIONS

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PURPOSE AND METHODS

Determination of neutron-gamma dose levels at various depths in animal and human phantoms is a necessary adjunct to radiobiological studies, to studies of radiation toxicity, and to the use of neutron sources for radiotherapy. Such measurements require small, sensitive, tissue equivalent dosimeters, as well as a knowledge of the physical and chemical parameters defining the tissues to be represented by the phantom. We have begun here by examining the applicability of some commercial dosimeters to the problems of neutron depth dosimetry, using various standard neutron and gamma sources to determine their accuracy, precision, energy response, and dose rate response. In addition, we have measured the densities and compositions of some of the tissues of radiobiological interest, using conventional gravimetric and combustion techniques.

For combustion analysis, tissues were dried to constant mass at room temperature over $MgClO_4$. Dried samples were then subjected to the usual C, H, N combustion, and ash residues were weighed and saved.

PROGRESS REPORT

In connection with their neutron dosimetry,⁽¹⁾ *T. castaneum* larvae were analyzed, as well as the flour media in which they are irradiated. Results are included in Table 50. Whole-body densities were measured by microscopic volumetric analysis for each of 13 weighed larvae, and a value of $0.949 \pm 0.008 \text{ g cm}^{-3}$ obtained.

Agar is used in compounding tissue equivalent gels for dosimetry phantoms,⁽²⁾ and is usually the only component whose composition is not known exactly from formula. Samples of the commonly used Bacto-Agar (Difco Inc., Detroit, Mich.) were analyzed and results are also included in Table 50. A 4% w/w gel of Bacto-Agar gave a measured density of 0.9866 g cm^{-3} at 25°.

* AUA-ANL Summer Bioengineering Student.

TABLE 50. CHEMICAL COMPOSITION OF SOME MATERIALS OF DOSIMETRIC INTEREST

Material ^(a)	H	N	O ^(b)	C	Cl	P	S	Ash
<i>T. castaneum</i> , larvae	9.36	3.15	60.24	25.90	0.06	0.33	0.11	0.82
<i>T. castaneum</i> , medium	6.71	2.25	50.10	40.47	0.07	0.17	0.13	0.10
Bacto-Agar ^(c)	5.77	0.21	44.51	45.25	0.19	0.00	0.90	3.17
Bone marrow ^(d)	10.15	3.41		13.11				
Depot fat	12.27	0.02		61.33				
Large intestine	10.29	2.83		11.01				
Small intestine	10.96	2.88		13.05				
Kidney	10.37	2.79		11.49				
Liver	10.13	3.02		12.42				
Skeletal muscle	10.24	3.29		11.98				

^(a) Analyses reported in weight percent. Analytical methods were precise to $\pm 0.02\%$.

^(b) By difference without allowance for O in ash.

^(c) Analysis reported on dry weight basis, H₂O was $19.50 \pm 0.70\%$.

^(d) All tissues pooled from 5 rats. Only C, H, N analyses completed to date.

TABLE 51. DENSITIES OF RAT AND MOUSE ORGANS AT 25°C

Organ	Mouse ^(a) , g cm ⁻³	Rat ^(b) , g cm ⁻³
Brain	1.0475 $\pm$ 0.009	1.0442 $\pm$ 0.0019
Skeletal muscle	1.0583 $\pm$ 0.0012	1.0562 $\pm$ 0.0013
Spleen	1.0826 $\pm$ 0.0011	1.0800 $\pm$ 0.0024
Kidney	1.0547 $\pm$ 0.0008	1.0566 $\pm$ 0.0010
Liver	1.0787 $\pm$ 0.0006	1.0725 $\pm$ 0.0013

^(a) Mean $\pm \sigma$ for 15 mice, reported previously.⁽²⁾

^(b) Mean $\pm \sigma$ for 5 SD/Anl[Anl-66] rats.

equivalent to a partial solution density, ⁽²⁾ ρ_a , of 0.788 g cm⁻³ for the agar.

Tissues from five female Sprague-Dawley rats, 320 days old and littermates weighing 280 ± 4 g, were subjected to analysis and to density measurements. Results are presented in Tables 50 and 51.

In addition, samples of skeletal muscle from the rats and from 15 CF#1 mice were measured for fat-free density by previously reported methods.⁽³⁾ The rats gave 1.065 and the mice 1.068, both in g cm⁻³ at 37°C.

Four other mice and one rat, taken randomly from the ANL Facility animal population and differing in age, strain, and sex were subjected to organ density measurements in a preliminary attempt to ascertain the probable spread of such values in a laboratory rodent population. Results are presented in Table 52.

In addition to the physicochemical studies above, we examined the suitability of some small, paired, commercial* ion chambers for depth dosimetry. Each pair

* Bendix Corp., Cincinnati 8, Ohio.

TABLE 52. DENSITIES OF VARIOUS RAT AND MOUSE ORGANS

Organ ^(a)	Mouse I, 27°	Mouse II, 27°	Mouse III, 26°	Mouse IV, 25°	Rat, 37°
Liver	1.0813	1.0874	1.0810	1.0779	1.0614
Femur	1.8715			1.6660	1.5812
Ovary					1.0665
Uterus	1.0685		0.0627	1.0684	1.0595
Eye lens	1.2216			1.2083	1.3470
Heart	1.0730	1.0515	1.0615	1.0736	1.0595
Small intestine	1.0417	1.0318	1.0254	1.0342	1.0356
Large intestine	1.0290	1.0432	1.0252	1.0289	1.0389
Stomach	1.0411		1.0442		1.0524
Lungs	0.6443	0.8243	0.8030	0.6848	0.8947
Pancreas	1.0663	1.3984			
Skin (shaved)	0.9591	1.0029			1.0715
Lymphatics	0.9736			1.0392	
Salivary glands	1.0538				
Whole eye				1.0748	
Bladder	0.97936			1.0338	
Thymus			1.03881		
Tumor (fibroma)					1.0515

^(a) Densities given in g cm⁻³ at the temperature noted.

TABLE 53. CALIBRATION OF SMALL TISSUE EQUIVALENT ION CHAMBER PAIRS

Source ^(a)	Pair I	Pair II	Pair III	Pair IV
Ra γ , tissue equivalent chambers	1.021 $\pm$ 0.013	1.196 $\pm$ 0.009	1.110 $\pm$ 0.016	0.995 $\pm$ 0.011
Ra γ , C-CO ₂ chambers	1.000 $\pm$ 0.010	1.202 $\pm$ 0.012	1.103 $\pm$ 0.009	0.945 $\pm$ 0.009
²⁵² Cf	0.97	0.99	1.02	0.97
Pu-Be	1.01	1.02	0.99	1.01
88 keV	1.08	1.09	1.09	1.07
398 keV	1.05	1.06	1.06	1.04
663 keV	1.05	1.07	1.08	1.05
891 keV	1.03	1.05	1.06	1.03
1321 keV	1.01	1.03	0.98	1.02

^(a) Values are given as fraction of the known dose from the calibration standard. Standard deviations for sources other than Ra were all about ± 0.02 .

consisted of a tissue equivalent chamber and a graphite-CO₂ chamber, both of the Rossi type.⁽⁴⁾ Full-scale reading was about 0.2 Rads, and sensitive volume was 1 cm³.

Eight pairs were selected and cross calibrated against a National Bureau of Standards radium γ source. Calibration was also effected by using the monoenergetic neutron facility previously described, a plutonium beryllium neutron source calibrated for us by NBS and a ²⁵²Cf source of exactly 5.00 μ g. C-CO₂ factors and equations were taken from the literature.⁽⁴⁾ Results are presented in Table 53.

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TISSUE EQUIVALENT PHANTOMS FOR STANDARD MAN AND MUSCLE

Norman A. Frigerio and Martin J. Sampson

PURPOSE AND METHODS

Although tissue equivalent chambers are widely used in experimental neutron dosimetry,⁽¹⁾ depth dose measurements require tissue-equivalent phantoms as well. Such phantoms impose more stringent requirements than do chambers with respect to elemental composition, chemical composition, density, and geometry.⁽²⁾ As phantoms meeting these requirements are not at present available, we endeavored to fill this need by using common physicochemical methods, taking the needed biological data from standard references.^{3,5} Currently, we have restricted ourselves to the two best characterized systems, Standard Man and lean, mammalian muscle.

PROGRESS REPORT

In addition to the requirements above, a tissue-equivalent liquid for use in phantoms should be moderate in cost, transparent, colorless, compounded of readily available substances, neither corrosive, toxic, volatile, nor unpleasant, and stable to radiation; also over a period of time it should not be subject to growth of microorganisms, to internal chemical reaction, or to absorption of CO_2 etc. from the air. Satisfying these requirements is quite an exercise in applied chemistry, and that process is too complex to describe in detail here. However, Table 54 lists the properties of a number of useful compounds, nearly any mixture of which will meet the requirements above so long as the system is chiefly aqueous. The only conspicuous exceptions are mixtures of the salts, especially Ca and Mg phosphates and sulfates. Where their concentrations are fairly low, as in muscle, one can resort to a mildly acidic medium or to the addition of a chelating agent (e.g., EDTA) to hold the ions in solution. At high concentrations, as in Standard Man, even chelates are insufficiently soluble, and we have been unable to avoid acidic solutions. We were also unable to avoid the use of a few uncommon compounds, although we were able to restrict ourselves to those both inexpensive and commercially available. With the exception of the fairly low pH of some of the

mixtures, then, they satisfy all of the requirements presented above.

Volatility is proportional to total equilibrium vapor pressure. This can be estimated by Raoult's Law;⁽²⁾

$$P = \sum_i X_i P_i, \quad (1)$$

where P is the total vapor pressure at a given temperature, X_i the mole fraction of the i th component and P_i its vapor pressure at the given temperature. In concentrated aqueous solutions, P will generally be lower than given by Equation (1) because of ionic and molecular interactions. For all of the mixtures given in this report, the total vapor pressure at 25° will be lower than that of pure water, even for mixtures containing some higher vapor-pressure components, because of the combined effects of Raoult's Law and of chemical interaction. Nonetheless, mixtures should be kept covered, both in use and in storage, to prevent slow changes in their composition and density through evaporation.

Similarly, density can be estimated from⁽²⁾

$$(\bar{\rho})^{-1} = \sum_i M_i \bar{\rho}_{a,i}, \quad (2)$$

where $\bar{\rho}$ is the mean density of the mixture, M_i is the weight fraction of the i th component, and $\bar{\rho}_{a,i}$ is its apparent specific density in solution.

Values of $\bar{\rho}_a$ are included in Table 54, some computed from literature data and some measured by us. With these and Equation (2) an estimate of $\bar{\rho}$ can be made to $\pm 1\%$ or so. In principle, the method of partial densities can be used to predict $\bar{\rho}$ to a few hundredths of a percent. In practice, however, this would require that $\bar{\rho}_a$ values be known for the exact concentrations and milieu used so that it is more practicable to use Equation (2) only for preliminary estimates, and take $\bar{\rho}$ by actual measurement on the final mixture. Where it is necessary to obtain a particular density, the amount of trial and error can be greatly reduced by making up two solutions, one of $\bar{\rho}$ slightly higher and one with $\bar{\rho}$ slightly lower than desired. These can then be combined linearly to give the $\bar{\rho}$ desired, as the error introduced by non-

TABLE 54. PROPERTIES OF SOME USEFUL COMPONENTS FOR TISSUE EQUIVALENT MIXTURES

Compound	Canonical formula	Apparent solvated density			Vapor pressure at 25°C, mm Hg	Solubility in H ₂ O at 25°C, moles/kg ^(a)	Formula wt, g/mole
		$\bar{\rho}_a$, ^(g) g cm ⁻³	Tempera- ture, °C	Concen- tration, moles/kg ^(a)			
Water	H ₂ O	0.997	25	55.5	23.76	∞	18.016
Propanol-1	C ₃ H ₈ (H ₂ O)	0.823 ^(e)	28	2.5	20.1	∞	60.094
Acetonitrile	C ₂ H ₃ N	0.831 ^(e)	25	3.5	92	∞	41.052
Ethylene glycol	C ₂ H ₂ (H ₂ O) ₂	1.112	20	1.6	0.1	∞	62.068
Ethylenediaminetetraacetic acid, (EDTA)	C ₁₀ N ₂ (H ₂ O) ₅	1.190 ^(e)	25	0.1 ^(b)	(f)	0.1 ^(b)	292.24
Glycerol	C ₃ H ₂ (H ₂ O) ₃	1.300	20	2.1	7.0	∞	92.094
Tris (hydroxymethyl) amino- methane, (TRIS)	C ₄ H ₉ N(H ₂ O) ₃	1.316 ^(e)	24	3.1	(f)	2.9 ^(e)	121.14
Urea	CH ₂ N ₂ (H ₂ O)	1.391	20	1.6	(f)	8.3	60.058
Malonomononitrile	C ₃ HON(H ₂ O)	1.318 ^(e)	24	2.8	(f)	3.1	85.062
Glucose	C ₆ (H ₂ O) ₆	1.654	20	0.55	(f)	2.7	180.156
Ca(H ₂ PO ₄) ₂ ·H ₂ O	CaO ₄ P ₂ (H ₂ O) ₃	3.73	19	0.1	(f)	0.07	252.09
Mg(NO ₃) ₂ ·6H ₂ O	MgO ₆ N ₂ (H ₂ O) ₆	3.84	20	0.1	(f)	1.64	256.43
KHSO ₄	KHSO ₄	3.13	20	0.2	(f)	4.1	136.17
NaCl	NaCl	3.38	20	0.4	(f)	4.55	58.45
NaH ₂ PO ₄ ·H ₂ O	NaPO ₃ (H ₂ O) ₂	3.83	25	0.1	(f)	1.2	138.01
H ₃ PO ₄ ·H ₂ O ^(d)	HPO ₃ (H ₂ O) ₂	2.719	20	0.2	3	∞	116.020
Ca(H ₂ PO ₂) ₂	CaP ₂ O ₂ (H ₂ O) ₂	3.18	25	0.5	(f)	0.78	170.06
1,5-Pentandiol	C ₅ H ₈ (H ₂ O) ₂	1.012 ^(e)	24	1.5	(f)	∞	104.146
Ca(NO ₃) ₂ ·4H ₂ O	CaO ₆ N ₂ (H ₂ O) ₄	3.64	25	0.3	(f)	3.8	236.160
MgCl ₂ ·H ₂ O	MgCl ₂ (H ₂ O) ₆	5.17	25	0.02	(f)	3.1	203.330
NaOH	NaOH	8.93	15	0.1	(f)	12.7	40.005
KOH	KOH	8.48	15	0.1	(f)	12.5	56.101
HCl:3.3 H ₂ O ^(d)	HCl(H ₂ O) _{3.3}	1.98	25	0.3	19	∞	95.918
Dimethyl sulfoxide	C ₂ H ₆ S(H ₂ O)	1.107 ^(e)	24	0.1	0.5	∞	78.134
HNO ₃ :1.5 H ₂ O ^(d)	NO _{2.5} (H ₂ O) ₂	2.11	25	1.0	13	∞	90.040
Triethylamine ^(h)	C ₆ H ₁₅ N	0.910	21	0.1	(f)	6.5	101.188
Acetic acid	C ₂ (H ₂ O) ₂	1.27	20	0.5	17	∞	60.052

(a) Moles solute per kg solution. Concentrations for $\bar{\rho}_a$ were chosen to be close to expectations for tissue equivalent mixtures.(b) In the presence of equimolar Ca⁺⁺ or Mg⁺⁺.(c) Although these solutions are somewhat alkaline, they will not absorb CO₂.(d) The composition of the usual commercial acid, i.e. 85% H₃PO₄, 79% HNO₃, 38% HCl.

(e) Values so marked were measured by us. The rest were obtained from the literature.

(f) Much less than 0.1 mm Hg.

(g) Values are per gram parent compound, not per gram hydrated form.

(h) Values given in acid solution; the free base is nearly insoluble in water.

linear combination of $(\bar{\rho})^{-1}$ values for the two will seldom exceed 0.01 %.

The difficulty in obtaining elemental compositions exactly matching their target values^(1, 2) experienced by previous workers suggests that a general method would be welcome. We, therefore, developed a simplified version of the Gibbs Method of Canonical Components,⁽²⁾ using water as an obligatory component with the individual elements the remaining canonical components, for solution of the problems of tissue equivalent composition.

This is illustrated in Table 55 for the simplest case—a tissue composition expressed in simple integers. Taking the well-known tissue approximation formula,⁽¹⁾ C₅H₄₀O₁₈N, we first transfer the canonically required

component H₂O, to yield C₅H₁₈N(H₂O)₁₈. Compounds of empirical formula C₅H₁₈N exist, but they meet few, if any, of the criteria presented above. Thus, we transfer sufficient moles of urea, a common compound that meets these requirements, to satisfy the needed one mole of N. By writing urea in canonical form, CH₂N₂(H₂O), it is evident that 0.5 moles are needed. This yields C_{4.5}H_{3.0} + 0.5 (CH₂N₂(H₂O)) + 17.5 H₂O. We now need a simple compound of C/H = 3/2, or, as H₂O is a canonically permitted component, (C₃H₂)_m(H₂O)_n. Glycerol is C₃H₂(H₂O)₃ so that transfer of 1.5 moles of glycerol yields 1.5(C₃H₂(H₂O)₃) + 0.5(CH₂N₂(H₂O)) + 13.0 H₂O = C₆H₁₀O₁₈N. The formula weight is 402.378 so, to make 1 kg of mixture, we take 1000/402.378 times as many moles of each com-

TABLE 55. FORMULATION OF SIMPLE, TISSUE EQUIVALENT MIXTURES

Component	C	H	O	N	H ₂ O	Urea	Glycerol	Ethylene glycol
C ₃ H ₄ O ₃ N, wt %	14.9	10.0	71.6	3.5				
Relative moles	5	40	18	1				
Transfer 18 H ₂ O	5	4	0	1	18			
Transfer 0.5 CH ₂ N ₂ (H ₂ O)	1.5	3.0	0	0	17.5	0.5		
Transfer 1.5 C ₃ H ₂ (H ₂ O) ₃	0	0	0	0	13.0	0.5	1.5	
Moles/kg, mixture					32.31	1.24	3.73	
g/kg, mixture					582.06	74.63	343.31	
Final wt %	14.90	10.00	71.60	3.50				
Earlier wt %	15.6	9.8	71.0	3.6				
Muscle equivalent, wt %	12.3	10.2	74.0	3.5				
Moles/kg	10.2415	101.1905	46.250	2.499				
Transfer 46.2500 H ₂ O	10.2415	8.6905	0.0	2.499	46.2500			
Transfer 1.2495 CH ₂ N ₂ (H ₂ O)	8.992	6.1919	0.0	0.0	15.0007	1.2495		
Transfer C ₃ H ₂ (H ₂ O) ₃ and C ₂ H ₂ (H ₂ O) ₂	0.0	0.0	0.0	0.0	36.0085	1.2495	2.8003	0.2956
Moles/kg, mixture					36.0085	1.2495	2.8003	0.2956
g/kg, mixture					648.73	75.03	257.89	18.35
Final wt %	12.30	10.20	74.00	3.50				
Earlier wt %	12.0	10.2	74.2	3.6				

ponent as indicated above. This yields a mixture of exact composition and, interestingly enough, of one less chemical component as well. The earlier mixture¹¹ utilized 569 g H₂O, 284 g glycerol, 76 g urea, and 71 g sucrose. For the density of the earlier mixture we obtained 1.112 g cm⁻³ at 25°. For the three-component mixture of Table 55, we estimated 1.0966 g cm⁻³ by Equation (2) and measured 1.1048 g cm⁻³, both at 25°.

When, as is usually the case, mole ratios are not integers, the first step is the reduction of the weight percent composition given to moles/kg. In Table 55, this is illustrated with a simplified muscle equivalent liquid previously described elsewhere.⁶ Again, water is first transferred; then 1.249 moles of urea are added to complete the N requirement. The C:H ratio remaining lies between 3, 2 and 1, e.g., between glycerol (*G*) at C₃H₂(H₂O)₃ and ethylene glycol (*E*) at C₂H₂(H₂O)₂. Setting up the simultaneous equations for carbon, 3*G* + 2*E* = 8.9922 and for hydrogen, 2*G* + 2*E* = 6.1919, we obtain *G* = 2.8003 and *E* = 0.2956. Estimated $\bar{\rho}$ = 1.0776, measured $\bar{\rho}$ = 1.0797 at 24°, earlier $\bar{\rho}$ = 1.07 at room temperature, all in g cm⁻³.

For more complex mixtures, the same procedure is applicable, provided that one first transfers components

containing minor elements (e.g., inorganic compounds), then H₂O, N, H, C, in that order. Where a high density mixture is desired, it is best to solve for H and N simultaneously, using high density components, then to satisfy C via transfer of glucose or acetic acid. This is because both C₆(H₂O)₆ and C₂(H₂O)₂ are canonically equivalent to pure C, and high in density as well. For low density mixtures it is best to solve C, H, N simultaneously, using low density components, so as to leave only C + H in ratio 1:2. This is because the lowest density components are generally of composition (CH₂)_{*m*}(H₂O)_{*n*}, e.g., propanol-1.

The composition of Standard Man has the force of an I.C.R.P. quantity, so that we have used it as given with only one small change, as shown in Table 56, increasing the oxygen by 0.7 % to bring the total to 100.00 %.

Taking density, sizes, shapes, volumes, etc. from literature⁽³⁻⁵⁾ we have also arrived at a geometric representation of the Standard Man shown in Figure 101 and described in Table 57. This phantom, filled with the Standard Man liquid of Table 58, should provide a useful simulacrum for either computation or experiment.

In compounding the mixtures of Table 58 the components should be added in the order indicated in the

TABLE 56. COMPOSITION OF THE TISSUE EQUIVALENT MIXTURES OF TABLE 58

Element	C	H	O	N	Ca	P	S	K	Na	Cl	Mg
Standard Man, wt %	18	10	65.7	3	1.5	1	0.25	0.20	0.15	0.15	0.05
Standard Man, moles kg ⁻¹	14.9875	99.2063	41.0625	2.1416	0.3743	0.3228	0.0780	0.0512	0.0652	0.0423	0.0206
Muscle, wt %	12.3	10.2	72.91	3.5	0.01	0.20	0.32	0.39	0.07	0.08	0.02
Muscle, moles kg ⁻¹	10.241	101.190	45.653	2.499	0.0025	0.0645	0.1000	0.1000	0.0304	0.0226	0.0082

TABLE 57. GEOMETRIC DESCRIPTION OF STANDARD MAN

	Position, cm (x, y, z)	Extension, cm ($\pm x, \pm y, \pm z$)	Volume cm ³	Geometric type
Head	(25.5, 10.5, 161)	(6.5, 8.5, 10)	3,471	Elliptical cylinder
Neck	(25.5, 10.5, 148.5)	(4.5, 5.5, 2.5)	389	Elliptical cylinder
Chest	(25.5, 10.5, 131)	(17, 11, 15)	17,624	Elliptical cylinder
Waist	(25.5, 10.5, 108)	(13.5, 9.5, 8)	6,447	Elliptical cylinder
Hips	(25.5, 10.5, 89)	(15.5, 10.5, 11)	11,248	Elliptical cylinder
Right leg	(14.75, 10.5, 39)	(6.5, 7.5, 39)	11,946	Elliptical cylinder
Left leg	(33.25, 10.5, 39)	(6.5, 7.5, 39)	11,946	Elliptical cylinder
Right arm	(4.5, 10.5, 118)	(4.5, 4.5, 28)	3,563	Circular cylinder
Left arm	(46.5, 10.5, 118)	(4.5, 4.5, 28)	3,563	Circular cylinder
Right lung	(18.5, 10.5, 131)	(6, 8, 11)	2,211	Ellipsoid
Left lung	(32.5, 10.5, 131)	(6, 8, 11)	2,211	Ellipsoid

Phantom coordinates are Cartesian, dimensions are in cm, and cylinders are right with axes parallel to the z direction. Total volume is 70.20 liters, inclusive of lung voids totalling 4.42 liters. Surface area is 1.85 m². Cartesian origin taken at lower left front corner of inclusive cube so that figure is entirely in the positive octant (see Figure 101).

FIG. 101. -Front and side views of an eleven-region Standard Man. Coordinate origin is indicated by the circled cross. Dimensions and positions are given in Table 57.

table, each component stirred into solution without heat before the next is added. In this way, it will be found that all components enter solution rapidly and completely at room temperature. Otherwise, intermediate precipitates may form, and these are often very difficult to bring back into solution.

In Table 58 a standard density mixture is given, along with mixtures of higher and lower density for those who might desire to obtain some other value by linear combination. Alternatively, inspection of the three mixtures indicates how other densities may be obtained at constant composition, by suitable choice of components from Table 54. Thus, increasing the ratio of triethylamine to HNO_3 , at constant percent N, decreases the H^+/C^- ratio needed in the final addition from 2.00 to 1.60. This shifts the requirement from the lower density compounds of $\text{H}^+/\text{C}^- \cong 2$, e.g., propanol, to the higher density compounds of H^+/C^- less than 2, e.g., pentanediol at $\text{H}^+/\text{C}^- = 1.60$. The other components are held constant because of the difficulty of obtaining an alternative salt system which is equally soluble and stable.

With few exceptions, the components of Table 58 are commonly available at such high purity and stability that no thought need be taken to their assay. For highest accuracy, however, assay values supplied with purchased HNO_3 , HCl and H_3PO_4 should be checked. If these are not, respectively, 70.0, 38.0 and 84.5% by weight, correction should be applied. Some samples of triethylamine or of malonomononitrile contain traces of water, as well as colored impurities which impart a yellow tinge to the final mixture. These may be removed by simple distillation for the triethylamine, and by recrystallization followed by *in vacuo* drying to constant weight for the somewhat hygroscopic malonomononitrile.

Elemental composition of lean muscle was taken from NBS Handbook 85⁽⁷⁾ for C, H, O, N, Ca, P and Mg, but K, Na and S were readjusted slightly⁽¹⁵⁾ to allow for the Cl omitted by Handbook 85. From these values three tissue equivalent mixtures of different density were formulated, as for Standard Man, and these are described in Tables 56, 58, and 59. The comments made for Standard Man apply, except that, though the EDTA

TABLE 58. COMPOUNDING OF TISSUE EQUIVALENT MIXTURES

Component	Standard Man						Muscle					
	Low ρ		Std. ρ		High ρ		Low ρ		Std. ρ		High ρ	
	wt %	moles kg ⁻¹	wt %	moles kg ⁻¹	wt %	moles kg ⁻¹	wt %	moles kg ⁻¹	wt %	moles kg ⁻¹	wt %	moles kg ⁻¹
H ₂ O	43.982	24.4128	47.692	26.4723	49.213	27.3165	74.065	11.111	62.160	34.502	66.220	36.756
Ca(H ₂ PO ₄) ₂ ·H ₂ O							0.0629	0.0025	0.0629	0.0025	0.0629	0.0025
Ca(NO ₃) ₂ ·4H ₂ O	8.838	0.3743	8.838	0.3743	8.838	0.3743						
MgCl ₂ ·6H ₂ O	0.418	0.0206	0.0206	0.0206	0.418	0.0206						
Mg(NO ₃) ₂ ·6H ₂ O							0.2109	0.0082	0.2109	0.0082	0.2109	0.0082
HNO ₃ ·1.5H ₂ O	12.215	1.3566	7.203	0.8000	7.155	0.7946						
HCl·3.3H ₂ O	0.012	0.0012	0.012	0.0012	0.012	0.0012						
H ₃ PO ₄ ·H ₂ O	3.745	0.3228	3.745	0.3228	3.745	0.3228	0.5997	0.0517	0.5997	0.0517	0.5997	0.0517
EDTA											0.877	0.0300
KHSO ₄							1.3617	0.1000	1.3617	0.1000	1.3617	0.1000
NaCl							0.1319	0.0226	0.1319	0.0226	0.1319	0.0226
NaH ₂ PO ₄ ·H ₂ O							0.1087	0.0079	0.1087	0.0079	0.1087	0.0079
NaOH	0.261	0.0652	0.261	0.0652	0.261	0.0652						
KOH	0.287	0.0512	0.287	0.0512	0.287	0.0512						
Triethylamine	0.369	0.0365	1.518	0.1500	6.056	0.5985						
TRIS											26.584	2.1944
Malonomononitrile							9.618	1.1308				
Urea			1.331	0.2216					7.454	1.2411	0.684	0.1139
Acetic acid											3.153	0.5250
1,5 Pentanediol			16.265	1.5618	23.413	2.2481						
Glycerol									2.604	0.2827		
Ethylene glycol									25.300	1.0761		
Propanol-1	29.271	4.871	11.821	1.9671			8.240	1.3712				
Dimethyl sulfoxide			0.609	0.0780								
Acetonitrile							5.615	1.3678				

TABLE 59. PROPERTIES OF THE TISSUE EQUIVALENT MIXTURES OF TABLE 58 AT 25°C^(a)

	Standard Man			Muscle		
	Low $\bar{\rho}$	Std. $\bar{\rho}$	High $\bar{\rho}$	Low $\bar{\rho}$	Std. $\bar{\rho}$	High $\bar{\rho}$
Computed $\bar{\rho}$, g cm ⁻³	1.053	1.072	1.092	1.011	1.078	1.100
Measured $\bar{\rho}$, g cm ⁻³	1.048	1.069	1.088	1.012	1.070	1.106
Measured $d\bar{\rho}/dT$, g cm ⁻³ deg C ⁻¹	0.00066	0.00063	0.00062	0.00067	0.00064	0.00061
Computed vapor pressure, mm Hg	23.0	23.4	20.6	21.9	20.3	21.9
Free water, wt %	51.157	55.171	54.869	74.266	62.361	66.421
Free water, computed mole fraction	0.830	0.926	0.956	0.910	0.857	0.912

(a) Computed values of $\bar{\rho}$, vapor pressure and mole fraction made without regard for ionization.

must be added before the TRIS, it will not dissolve completely until after TRIS addition. In the muscle mixtures density increase was obtained by shifting from a trio of compounds of low combined density (acetonitrile, malonomononitrile, propanol), to a system based almost entirely on a single high density, alkaline compound (TRIS). The chelating agent EDTA was added to counter the salt precipitation effect of the increased alkalinity, and acetic acid is used as a canonical carbon component to lower the pH to 5.5.

CONCLUSIONS

As the maximum range of soft tissue densities and compositions lies well within the range of useful compounds given in Table 54, it appears that any real tissue can be matched by tissue equivalent liquid, using the methods outlined above. It now becomes necessary to obtain composition, density, and geometry data for other organs and tissues so that tissue equivalent systems can be devised for them as well. This requires a combination of literature evaluation and experiment,

both of which are under way in our laboratories. Not all experimental requirements can be met by liquids, however, so that efforts are also being made to provide tissue equivalent gels and plastics of appropriate mechanical properties for phantoms and ion chambers, using previously proposed chemical systems and reactions. Measurements of chemical binding and neutron optical effects in tissues are also under way. Thus, it appears probable that systems which are macroscopically tissue equivalent for neutrons of all energies will soon become available.

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DOSIMETRY OF SMALL ^{252}Cf SOURCES IN A HUMAN PHANTOM

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PURPOSE AND METHODS

Interest in use of implantable ^{252}Cf sources for the fast neutron therapy of cancer has increased.^(1, 2) Because precise knowledge of the n/γ dose distribution from such sources is a prerequisite to therapeutic trials, we attempted to satisfy this need as part of our program on the application of neutrons to cancer therapy. Although a similar study has been published,⁽¹⁾ the responses of Si diode and foil detectors used were so different from tissue equivalence,⁽³⁾ and the probability of considerable error was so large, that a redetermination of dose distributions with tissue equivalent systems seemed warranted.

To make these measurements, the central 25 cm of a commercial human phantom† was replaced with a Lucite-walled tank of the same shape, filled with the tissue equivalent liquid used previously.⁽¹⁾ A 5.00- μg ^{252}Cf source was positioned in the center of this section, and tissue equivalent and gamma sensitive chambers were arranged about it at various distances. Sources, chambers, and calibration methods were the same as those described for other dose measurements.⁽¹⁾

Program BIM-130⁽⁵⁾ was used to compute the n/γ dose distribution in a homogenous volume of the same elemental composition, density, and shape as the experimental tank. This program provides much more information about the space-energy distributions of the n/γ interactions and of LET spectra than can be obtained by experiment. Because of this, it was hoped that agreement between computation and experiment would

justify the use of the program to interpolate and extrapolate dose and LET distributions in regions where these cannot be measured; such agreement has been obtained in other comparisons of BIM-130 with experiment.^(5, 6) In computing the dose, the ^{252}Cf n and prompt γ spectra used were taken from the literature,^(7, 8) and the fission product spectrum was assumed to be the same as that of ^{235}U at equilibrium.⁽⁴⁾

PROGRESS REPORT

Figures 102-104 present results obtained for 1.00 μg ^{252}Cf compared with the data of Oliver and Wright.⁽¹⁾ A measurable discrepancy between theory and results was expected from the simplified computation employed, i.e., without regard for the presence of the dosimeters themselves. Some mechanical difficulty was also encountered in positioning the dosimeters to the ± 0.1 mm needed for precise dosimetry; therefore, the experimental figures are less precise than the computed ones. Despite this problem, good agreement was obtained between our experiments and the BIM-130 computations.

Differences between our results and those of Oliver and Wright⁽¹⁾ are probably related to their use of the non-tissue-equivalent Si diodes. These diodes were calibrated against a bare reactor spectrum and then used with a quite different moderated ^{252}Cf spectrum. They show an uncertainty exceeding $\pm 50\%$ in this energy region,⁽³⁾ so that the discrepancy observed is not surprising. As to the γ difference, we note that the previous workers obtained a n/γ dose ratio of 1.45 for the bare source, while ours was 2.36. As the n/γ ratio for a ^{252}Cf source will increase with time after preparation

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FIG. 102. Neutron dose rate as a function of distance from the source.

FIG. 103. Gamma dose rate as a function of distance from the source.

FIG. 104. Total dose rate as a function of distance from the source.

because of the approach to fission product equilibrium, their source probably had not attained equilibrium when their measurements were made. In support of this, ^{252}Cf measurements made at another laboratory⁽²⁾ using Si diodes calibrated against a tissue equivalent chamber gave dose rates intermediate between ours and those of Oliver and Wright.

CONCLUSIONS

It appears that even a relatively simple system will give good agreement between experimental ^{252}Cf dosimetry and the predictions of the BIM-130 program. Thus, we are encouraged to extend our measurements, using a more precise mechanical positioning system which we have recently completed to greater penetrations and to more complex organ, bone, and soft tissue phantoms. If these continue to show good agreement with BIM-130 computations, the latter will be used to describe completely the dose-space-particle energy-LET distributions from ^{252}Cf sources in therapeutically useful situations, so that the potential of such sources for cancer therapy can be fully utilized.

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QUANTITATIVE DETERMINATION OF PEROXIDE IN TUMOR TISSUE

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PURPOSE AND METHODS

Several lines of evidence have suggested an abnormally high concentration of peroxide in tumor tissue. In testing boronic acids for possible use in neutron capture therapy,⁽¹⁾ it was noted that borono Evans blue was oxidatively hydrolyzed *in vivo* to free H_3BO_3 .⁽²⁾ Additional investigation showed that this hydrolysis only occurred in the tumor-bearing mice and could not be confirmed for other laboratory mammals or for humans.⁽³⁾ This reaction could have been catalyzed enzymatically, and a search for such an enzyme began.⁽²⁾ A second possible explanation for the above reaction is the presence of peroxide, for it is known that benzeneboronic acids are oxidized to borate esters by H_2O_2 and the esters subsequently hydrolyzed to phenol and H_3BO_3 .⁽⁴⁾

In normal tissue, the concentration of H_2O_2 never reaches a level high enough to promote the above reaction because of the presence of catalase. Tumor tissues, however, are generally known to have a reduced activity of catalase,⁽³⁾ and so there is the possibility of a high H_2O_2 concentration. Subsequently, two other investigations have been made of the interrelationships of peroxide and boronic acid in tissue systems.

The first was a study of the hydrolysis of a benzeneboronic acid in plant and animal tissue homogenates by measurement of the amount of phenol produced.⁽⁶⁾ No oxidative hydrolysis of benzeneboronic acids was found in the normal tissues tested. But such activity was found in all three of the spontaneous tumor homogenates tested at a level equivalent to 10^{-4} *M* H_2O_2 , which is at least a hundred times that in other tissues.

In the second study,⁽⁷⁾ a colorimetric micromethod for the determination of H_2O_2 and similar peroxides

was developed, using *meta*-nitrophenylboronic acid (NPBA), and the method was applied directly to tissue systems.⁽⁸⁾ Qualitatively, the results confirmed the previous investigation, but a quantitative determination could not be obtained because of the difficulty in clarifying the tissue extracts. As a continuation of these preliminary studies, a more extensive study was undertaken to work out an NPBA method that could be applied to tissue systems in a quantitative manner, and to test a larger number of neoplastic tissues for their ability to hydrolyze NPBA.

The NPBA method was developed by using various normal tissues containing known concentrations of added H_2O_2 and by measuring the amount recovered by the NPBA reaction. Centrifugation and precipitation techniques produced turbid, red to yellow solutions. Dialysis, on the other hand, produced clear solutions with no color from extraneous tissue compounds. We also found that completing the process at low temperature (1° to 3°C) and using sand, mortar, and pestle instead of a homogenizer helped to avoid denaturation of the proteins which seemed to destroy most of the H_2O_2 , probably by unmasking of $-\text{SH}$ groups.

Weighed samples of normal or tumor tissues were soaked in a solution of NaN_3 ($4 \times 10^{-2} M$, 0.5 ml) to inhibit any catalase and then ground in a mortar and pestle with sand until the tissues were thoroughly homogenized (about 5 min). NPBA ($10^{-2} M$, 1.0 ml) was then added to the tissue homogenate, and the mixture was dialyzed against 12 ml distilled water for 8 to 10 hr. After dialysis, 0.1 ml of 19 M NaOH (2 drops) was added to the dialysate to produce the yellow color that is characteristic of the nitrophenoxide ion:

colorless

yellow

The absorbances of the solutions were read at 400 $m\mu$ in 5.0 cm cuvettes on a Beckman DU spectrophotometer.

As tissue mass, dialyzing volume, dialysate volume, and absorptivity coefficients were known, the initial H_2O_2 concentration in the tissue could be calculated.

PROGRESS REPORT

A number of normal tissues and spontaneous tumors from rats and mice were tested by the above method, and the results are shown in Table 60. Liver, spleen, brain, intestine, lung, kidney, skin, muscle, and heart from each of 18 control animals were tested but gave no suggestion of H_2O_2 . However, as seen from Table 60, seemingly normal tissues from tumor bearing animals gave some indication of H_2O_2 , and tumors gave very high concentrations of H_2O_2 . That the reactant was H_2O_2 is shown by the experiment of Table 61. Here, tumor dialysates obtained in the presence of NaN_3 but absence of NPBA gave reactions characteristic of H_2O_2 ,^(a) including the specific Ti complex.⁽⁹⁾ Even though oxidation of Fe^{++} or I^- in strong acid is not strictly specific for H_2O_2 , it is difficult to imagine any tissue oxidants other than peroxides strong enough to bring about these reactions. Certainly no biochemical systems are known whose oxidation potentials equal those of the Fe^{++} (0.777 volts) or I^- (0.540 volts) systems.

Tumor dialysates obtained in the absence of both NaN_3 and NPBA gave little H_2O_2 , suggesting the action of, e.g., blood catalase. Tumor dialysates obtained in the presence of NPBA but absence of NaN_3 gave about half the H_2O_2 yield of dialysates from other samples of the same tumor tested by the complete method. This suggests the NPBA competes fairly successfully with catalase for H_2O_2 , possibly by inhibition of the enzyme.

The fact that the oxidant was dialyzable and gave tests for H_2O_2 rules out the possibility of an enzymatic hydrolysis of the NPBA. The fact that NPBA and NaN_3 are strong bacteriocides,⁽¹⁰⁾ along with the low temperature, rules out the possibility of a bacterial action specific to tumor tissue. Two other possibilities exist: 1) a high H_2O_2 concentration, *in vivo*, in tumors; 2) a chemical system unique to tumors and capable of producing H_2O_2 on reaction with air [either enzymatic (e.g., flavoenzymes) or autocatalytic (e.g., cysteine)]. Treatments which denatured tissue proteins (e.g., heat, extremes of pH) not only prevented detection of H_2O_2 but even destroyed added H_2O_2 . Thus, the autoxidation of cysteine, etc., is ruled out because such reactions are promoted by heat and by high pH rather than inhibited.

CONCLUSIONS

A series of spontaneous rat and mouse tumor homogenates have all shown H_2O_2 concentrations two to three orders of magnitude greater than tissues from

TABLE 60. H_2O_2 IN TISSUES FROM TUMOR BEARING ANIMALS

Animal	Tissue ^(a)	H_2O_2 , $\mu\text{M}/\text{kg}$ tissue	
		Standard NPBA method	Modified method
Mouse I, C3H/HeJ	Muscle	83	70 ^(b)
	Mammary tumor	400	315 ^(b)
	Metastasis	650	600 ^(b)
Mouse II, C3H/HeJ	Muscle	0	
	Mammary tumor	730	705 ^(b)
Mouse III, CF #1, Anl [Anl 66]	Muscle	250	
	Fibrosarcoma	340	
Mouse IV, CF #1 Anl [Anl 66]	Muscle	0	
	Lymphoma	340	
Rat V	Muscle	0	
	Fibroma	680	590
Rat VI	Muscle	0	
	Fibroma	770	700 ^(b)
Rat VII	Liver	28	10 ^(b)
	Hepatoma	940	900 ^(b)

^(a) Non tumor tissues taken from near the tumor.

^(b) NPBA added to dialysate after dialysis.

TABLE 61. COMPARISON OF H_2O_2 REACTIONS

Method	Acid I^-	$\text{Fe}^{++} + \text{SCN}^-$	Ti^{++}	NPBA
H_2O_2 , moles liter ⁻¹	5.1×10^{-8}	5.4×10^{-5}	4.9×10^{-5}	5.2×10^{-5}

Tumor dialysate from Rat VI of Table 60 used for all determinations; NaN_3 only present during dialysis.

healthy animals. If this truly reflects a high *in vivo* tumor concentration of H_2O_2 , a number of new chemotherapeutic avenues are opened. For example, an alkylating agent could be held in sterically hindered form by carbon—boron—carbon bonds. The latter would cleave only in the presence of H_2O_2 , releasing the cytotoxic alkylating groups in, and only in, the tumor. If, on the other hand, the high H_2O_2 concentrations found are due to an *in vitro* oxidation peculiar to tumor tissue, elucidation will be necessary before the possible therapeutic implications can be formulated. Work is currently under way to resolve this question. As an aid in this work we were recently able to synthesize the *para* analog of the *meta*-NPBA used above. This reacts in the same way as *meta*-NPBA but gives a seventeen-fold increase in sensitivity as a consequence of the much

higher absorptivity of the *para*-nitrophenoxide ion produced.

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LEAST SQUARES ADJUSTMENT OF HYDROLYSIS DATA FOR ANTITUMOR COMPOUNDS OF PLATINUM (II)

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PURPOSE AND METHODS

The recent utilization of certain chloroammine and chloroamine complexes of platinum(II) as antitumor agents⁽¹⁾ stimulated our interest in the aquation equilibria of such compounds. In aqueous solutions at pH values of biochemical interest, such compounds undergo successive aquation reactions as indicated by Equations (1) and (2):

where Λ_2 is two ammine ligands, two amine ligands, or the bidentate ligand ethylenediamine (en). The concentration equilibrium quotients for the above reactions, K_1 and K_2 , are related to the total complex concentration, A , and to the equilibrium chloride ion concentration, T , by the expression⁽²⁾

$$T^3 + K_1T^2 + (K_1K_2 - AK_1)T - 2AK_1K_2 = 0. \quad (3)$$

The aquo complexes of the reactions of Equations (1) and (2) are weak acids, titratable with NaOH. Because it is generally only possible to titrate one proton of each water ligand, the equilibrium acid concentration is equal to the equilibrium chloride ion concentration. Thus, titrations of equilibrium solutions provide a basis for the evaluation of K_1 and K_2 .

Although Equation (3) can be solved for K_1 and K_2 from titration data for equilibrium solutions of any two different total complex concentrations, the least squares

method outlined in this report, when applied to data from solutions of six different concentrations over a ten-fold total complex concentration range, provided values for K_1 and K_2 with an order of magnitude increase in accuracy.

Equation (3) is linear in the two parameters, $\alpha = K_1$ and $\beta = K_1K_2$, and could be treated as a function of the form $Y = \beta X + \alpha$. Such treatment, however, would involve variables of little physical significance. Furthermore, both variables would be functions of T , which would force one to consider both X and Y subject to error. For the treatment described in this report, a generalized least squares adjustment, such as that described by Deming,⁽³⁾ has been applied to Equation (3) directly. The A variable has been assumed to be exact, a simplification well justified because the errors in total complex concentrations, A , (determined by dissolving weighed quantities of solid compound) were at least an order of magnitude smaller than the errors in the T variable.

PROGRESS REPORT

A computer program was written to perform the desired least squares adjustment. The quantity that was minimized was the sum of the weighted squares of the differences between the experimentally determined and the least squares adjusted values for T , i.e.,

$$S = \sum_i W_i (T_i^{\text{exptl.}} - T_i^{\text{calc.}})^2. \quad (4)$$

The program was written to output the values of K_1 and K_2 with their associated standard deviations. Also,

TABLE 62. TITRATION DATA FOR THE DETERMINATION OF K_1 AND K_2 FOR $[\text{PtCl}_2(\text{en})]$, IONIC STRENGTH = 0.318

Initial $[\text{PtCl}_2(\text{en})]$, millimolar	$T^\circ\text{C}$	Equilib. acid concentration, millimolar	
		Exptl. $\pm \sigma$	Calcd.
1.0019	35.0	0.8913 ± 0.0054	0.8909
0.80152		0.7536 ± 0.0031	0.7544
0.60114		0.6093 ± 0.0036	0.6066
0.40076		0.440 ± 0.004	0.4435
0.20038		0.257 ± 0.002	0.2562
0.10019		0.145 ± 0.002	0.1456
1.0060	30.0	0.8742 ± 0.0041	0.8728
0.80483		0.7426 ± 0.0043	0.7424
0.60362		0.5994 ± 0.0038	0.6003
0.40242		0.439 ± 0.003	0.4419
0.20121		0.258 ± 0.001	0.2576
0.10060		0.147 ± 0.002	0.1470
1.0008	25.0	0.8589 ± 0.0045	0.8572
0.80064		0.7287 ± 0.0062	0.7299
0.60048		0.5882 ± 0.0043	0.5909
0.40032		0.436 ± 0.003	0.4357
0.20016		0.255 ± 0.003	0.2545
0.10008		0.146 ± 0.002	0.1455

by utilization of a subroutine that employs a Newton-Raphson iterative technique,⁽⁴⁾ the program solves the cubic equation for the values of T at each experimental value of A , using the least squares values of K_1 and K_2 . The percent differences between such calculated values for T and the experimentally determined T values were less than one percent of the calculated values in most cases. The program also generates a plot of calculated T values vs. A values and superimposes the (A, T) data points. The required input information consists of the (A, T) data points, the experimental standard deviation of each T value, and initial estimates for K_1 and K_2 .

Typical titration data for the dichloro(ethylenediamine)platinum(II) complex are presented in Table 62. Each solution was titrated ten times, and the experimental standard deviation, σ , has been included. The calculated values in this table are the least squares adjusted values obtained by minimization of the summation of Equation (4). A typical least squares plot appears in Figure 105.

CONCLUSION

From these results, together with the results of similar adjustments at the same temperatures, the values of Table 63 were obtained for the concentration equilib-

FIG. 105.- Least squares plot of Equation (3) with the (A, T) data points superimposed for the 35.0°C data of Table 62. A and T are in millimolar units.TABLE 63. SUMMARY OF EXPERIMENTALLY DETERMINED VALUES FOR THE CONCENTRATION EQUILIBRIUM QUOTIENTS, K_1 AND K_2 , FOR THE SUCCESSIVE AQUATION REACTIONS OF $[\text{PtCl}_2(\text{en})]$

Constant	Value	Temperature
$K_1 \times 10^6 M$	2.76 ± 0.05	35.0°C
	2.33 ± 0.09	30.0°C
	2.19 ± 0.11	25.0°C
$K_2 \times 10^4 M$	1.38 ± 0.05	35.0°C
	1.48 ± 0.05	30.0°C
	1.43 ± 0.11	25.0°C

$$\Delta H^\circ_1 = 4 \text{ kcal mole}^{-1}; \Delta H^\circ_2 = -1 \text{ kcal mole}^{-1}.$$

rium quotients associated with the successive aquation reactions of $[\text{PtCl}_2(\text{en})]$. It is clear that this complex is largely aquated in aqueous solution, so that the nature of the anionic moiety (Cl, Br, etc.) will probably have little influence on antitumor activity.

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CERENKOV RADIATION AND LIQUID SCINTILLATION COUNTING

Walter E. Kisielewski

PURPOSE AND METHODS

Counting Cerenkov radiation to measure energetic β emitters commonly used in biology and medicine (^{32}P , ^{131}I , ^{24}Na , ^{90}Y , ^{42}K , and ^{47}Ca) is a recent development in liquid scintillation methodology.⁽¹⁻³⁾ Cerenkov radiation is produced when a charged particle (e.g., β particle) passes through a transparent medium at a velocity greater than the speed of light in the same medium. It is characterized by a bluish-white light emission that is directional, relatively weak in intensity, and has a continuous spectrum whose energy is concentrated mainly in the ultraviolet, but extends into the visible and becomes negligible in the infrared and microwave region. In water, which is an optically denser medium than air, the lower energy limit of electrons for the stimulation of Cerenkov radiation has been determined to be 0.265 MeV.⁽⁴⁾

Compared to conventional liquid scintillation procedures, sample preparation is relatively simple because completely aqueous systems can be counted without the addition of organic fluors. The counting efficiency is unaffected by chemical quenching; however, color and ultraviolet quenching are still a major problem.

TABLE 64. EXPERIMENTAL CERENKOV COUNTING EFFICIENCIES

Nuclide	E_{max} (MeV)	Counting efficiency (%) of disintegration)
Calcium-47	0.66 (83%), 1.94 (17%)	8.0
Potassium-40	1.32 (89%)	16.0
Sodium-24	1.39 (100%)	21.0
Phosphorus-32	1.71 (100%)	30.7
Potassium-42	2.0 (18%), 3.6 (82%)	64.0

TABLE 65. EFFICIENCY OF COUNTING ^{32}P CERENKOV RADIATION IN VARIOUS MEDIA

Solvent	Counting efficiency (%) of disintegration)
Water	30.7
Toluene	32.4
Ethanol/water (1:1, v/v)	31.5
H_3PO_4 (4N)	30.4
HNO_3 (6N)	29.6
Ethanol, H_3PO_4 (1:1, v/v)	30.8
Chloroform/methanol (2:1, v/v)	33.4
Formic acid (98%, w/w)	30.1

The purpose of this investigation was to examine selected experimental parameters that determine Cerenkov response to β -emitting radionuclides in liquid counting systems and to assess their application to biological and medical research.

Major interest was that Cerenkov techniques might be used in combination with liquid scintillation counting methods⁽⁵⁾ to measure and detect energetic β emitters (^{32}P , ^{131}I , and ^{42}K) in the presence of low energy β emitters (^{33}P , ^3H , and ^{14}C).

PROGRESS REPORT

A most important criterion in the utilization of Cerenkov radiation for radionuclide assay is β detection efficiency. In Table 64 experimental Cerenkov counting efficiencies are presented for a number of β emitters of different energy. To obtain these measurements, a Beckman LS-100 ambient temperature spectrophotometer was used and the counter was optimized to give maximum counting performance. Samples were prepared in glass scintillation counting vials, and appropriate aliquots of each radionuclide were added to 10 ml of distilled water. The results indicate that the Cerenkov detection efficiency for β particles is a direct function of their energy.

We then investigated the counting efficiency of ^{32}P ($\text{Na}_2^{32}\text{PO}_4$) by Cerenkov radiation in the presence of various solutions of salts, acids, and bases (media). These results are shown in Table 65. Cerenkov radiation in aqueous samples is not diminished by colorless acids, alkalis, or salts. The decrease in counting efficiency with nitric acid is due entirely to color quenching.

In acidity studies (Figure 106) phosphoric acid, regardless of pH values, gave stable counting rates; this was not true of nitric acid. A decrease in counting efficiency was noted at higher molarities, again, attributed to color quenching.

Results of the effect of sample volume on Cerenkov counting efficiency, determined for solutions of phosphoric and nitric acid in our system, are shown in Figure 107. The efficiency of Cerenkov detection as seen in Figure 107 is a function of the total sample volume. In the system used in this study, efficiency is maximum at a sample volume of 10 ml. Identical counting volumes must be used to obtain reproducible counting efficiencies when various samples are to be compared.

Measurements were also made to determine and

FIG. 106. The measurement of Cerenkov radiation as a function of the acidity of H_3PO_4 and HNO_3 .

compare counting efficiencies for ^{32}P , ^{33}P , and ^3H by Cerenkov detection and liquid scintillation counting with organic fluors.⁽⁶⁾ Various concentrations (dpm/ml) of ^{32}P , ^{33}P , and ^3H were added to counting vials containing 4.0 ml of phosphoric acid in alcohol solution. Each sample was first counted directly by the Cerenkov technique, after which 15 ml of a dioxane-based liquid scintillation fluor was added to each vial, which was recounted by the standard procedure for liquid scintillation measurement. The results shown in Table 66 clearly indicate that in dual-labeled experiments this combined method of analysis allows the quantitative measurement of energetic β emitters in the presence of low energy β emitters as ^3H , ^{14}C , and ^{33}P .

CONCLUSIONS

1. From the experimental results obtained, the most direct advantage of Cerenkov counting is the simplicity of sample preparation.

2. Samples can be counted in a completely aqueous system without adding scintillating fluors.

3. The economy, based on both simplicity of procedure and cost of fluor reagents, favors acceptance for large-scale analyses. Although chemical quenching appears to be completely eliminated, color and ultraviolet quenching is a problem.

4. Of prime importance: dual-labeling experiments that require energetic β emitters (^{32}P) in the presence of lower energy β emitters (^3H , ^{14}C , and ^{33}P) now can be successfully undertaken without recourse to radiochemical separation.

Cerenkov counting can be a useful method for the

FIG. 107. The measurement of Cerenkov radiation as a function of the volume of two acidic media.

TABLE 66. COUNTING EFFICIENCIES FOR ^{32}P , ^{33}P AND ^3H

Volume of each nuclide ^(a)	Cerenkov counting, cpm			Liquid scintillation counting, cpm		
	^{32}P	^{33}P	^3H	^{32}P	^{33}P	^3H
50 μl	30,113	135	bkg	65,931	23,713	22,134
100 μl	58,086	296	55	126,749	45,571	40,017
200 μl	120,264	574	121	262,372	94,672	78,389
Counting efficiency, %	30.7	0.53	0.029	67.3	87.4	22.1

^(a) 100 μl of ^{32}P = 192,374 dpm

100 μl of ^{33}P = 53,151 dpm.

100 μl of ^3H = 191,000 dpm

bkg = background = 36 cpm.

measurement of energetic β emitting radionuclides—especially in biological samples. Cerenkov counting technique in combination with conventional liquid scintillation procedures offers a new dimension in liquid scintillation methodology.

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GENETICS OF THE "MOTTLED" ALLELES ON THE X-CHROMOSOME OF THE MOUSE

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PURPOSE AND METHODS

We have been studying the genetics of the "mottled" alleles as an adjunct to investigations of the radiation induced mutation rate for sex-linked detrimental genes.^(1,2) Several genes in the allelic series are being used in those experiments as marker genes. The alleles under investigation are: blotchy, *Blo*; brindled, *Mo^{br}*; dappled, *Mo^{dp}*; tortoise, *To*. These genes vary in their lethal effects, both before and after birth, in their pathologic manifestations, and in their phenotypic expression of mosaic coat color.

The data are derived from stock maintenance records, from breeding records, from the mutation-rate studies, and from special matings to test interallelic behavior. All animals are sexed at birth and phenotypically classified at that time, if possible, or as soon as development permits.

PROGRESS REPORT

Phenotypic Variation

The mottled genes act on coat color by an interference in pigment production. Affected hairs are grey to white, but some collection of black pigment granules will often be seen in the tips of the long guard hairs. This reduces the intensity of depigmented areas and, in viable males, as blotchy and occasionally brindled, and otherwise near-white coat will have a stippled dark overcast.

Brindled, dappled, and tortoise have nearly identical phenotypes; only dappled is differentiated by depigmented spots in the ears and tail. The mosaic depigmentations in the coats of these three alleles are similar. The random mosaic expression is attributed to the random inactivation of one or the other X-chromosome, and all the genes contained thereon, early in development.⁽³⁾ When the X-chromosome carrying the wild-type allele is inactivated, the mutant phenotype is seen.

The gene *blotchy* is much less expressive than the others. A carrier female is sometimes indistinguishable from the wild-type.

The genes vary in viability effects, but only *Blo* is normally viable in the male or homozygous female.

Reproductive Performance

The basic reproductive capabilities of these alleles are presented in Table 67. The upper half of the data is derived from standard matings of the carrier dam with a wild-type sire and data in the lower half of the table are from carrier dams top-crossed with *Blo* males. The important features to be noted are the normal viability of all *Blo* genotypes, the postnatal male lethality for *Mo^{br}* along with nearly normal viability for the females, the complete prenatal male lethality for *Mo^{dp}* and *To* accompanied by some reduced prenatal and postnatal viability in these carrier females. Of the allelic combinations with *Blo*, *Blo Mo^{br}* is sublethal postnatally, but *Blo Mo^{dp}* and *Blo To* are essentially postnatal lethals. One *Blo To* female survived to 105 days of age. A point worth noting in all of these data is the genetically predictable behavior of the sex ratio; it drops from 0.5 to 0.33 for the prenatal male lethals. This adherence to expectation is not always seen, as among the progeny of XO dams,⁽⁴⁾ and it confirms the lethal gene effect as a zygotic lethal, not a gametic lethal.

We also have been breeding the *Blo Mo^{br}* female on the long shot that a recombinant wild-type might occur, because this locus seems to be a complex one with considerable intraallelic differentiation. None has occurred in over 500 offspring. The data indicate, however, a normal fecundity but a reduced survival among the progeny suggestive of a deficiency in milk production.

Relative Viability

Table 68 defines the relative viability among the four alleles and the available combinations according to seven classes of pre- and postnatal lethality, sublethality, and viability. The availability of a fertile *To* male, reported earlier,⁽⁵⁾ permitted some otherwise forbidden allelic tests although *To/To* was, itself, not tested. Although this classification uses somewhat categorical subclassification, it should be noted that the time of death is actually a continuously distributed property between conception and

TABLE 67. REPRODUCTIVE PERFORMANCE AMONG ALLELES IN THE MOTTLED SERIES

Genotype		N	Litter size	Sex ratio	Ratio born				Weaned, %			
Dam	Sire				+/+	Mo/+	+ y	Mo Y	+/+	Mo/+	+ Y	Mo Y
<i>Blo</i> /+	+ Y	624	7.6	0.49	1.0		0.97		86		86	
<i>Mo^{br}</i> /+	+ Y	477	8.2	0.53	1.0	1.10	1.24	1.03	93	83	92	0
<i>Mo^{dp}</i> /+	+ Y	776	5.7	0.37	1.0	0.69 ^(a)	1.16	(0) ^(b)	75	77	71	-
<i>To</i> /+	+ Y	626	5.4	0.35	1.0	0.82 ^(a)	0.98	(0) ^(b)	91	61 ^(a)	85	-
					<i>Blo</i> /+	<i>Blo</i> / <i>Mo</i>	+ Y	<i>Mo</i> Y	<i>Blo</i> /+	<i>Blo</i> / <i>Mo</i>	+ Y	<i>Mo</i> Y
<i>Blo</i> /+	<i>Blo</i> Y	105	6.6	0.58	1.0	0.8	1.5	1.1	91	100	97	96
<i>Mo^{br}</i> /+	<i>Blo</i> Y	148	7.4	0.50	1.0	1.2	1.3	1.0	100	59	93	0
<i>Mo^{dp}</i> /+	<i>Blo</i> Y	110	5.8	0.33	1.0	0.9	0.9	0	77	0	83	-
<i>To</i> /+	<i>Blo</i> Y	135	5.9	0.36	1.0	0.7	0.9	0	100	3	91	-

^(a) Difference from +/+ class significant at $P < 0.05 > 0.01$.^(b) These classes of males normally die *in utero*, but occasional individuals are stillborn.

TABLE 68. RELATIVE VIABILITY OF MOTTLED GENOTYPES

	Sperm			
	+	<i>Blo</i>	Y	<i>To</i>
<i>Blo</i>	(7) <i>Blo</i> /+	(7) <i>Blo</i> / <i>Blo</i>	(7) <i>Blo</i> Y	(2) <i>Blo</i> / <i>To</i>
<i>Mo^{br}</i>	(7) <i>Mo^{br}</i> /+	(6) <i>Mo^{br}</i> / <i>Blo</i>	(2) <i>Mo^{br}</i> Y	(2) <i>Mo^{br}</i> / <i>To</i>
<i>Mo^{dp}</i>	(4) <i>Mo^{dp}</i> /+	(2) <i>Mo^{dp}</i> / <i>Blo</i>	(1) <i>Mo^{dp}</i> Y	(1) <i>Mo^{dp}</i> / <i>To</i>
<i>To</i>	(3) <i>To</i> /+	(2) <i>To</i> / <i>Blo</i>	(1) <i>To</i> Y	(1) [<i>To</i> / <i>To</i>]
Class	Prenatal		Postnatal	
1	lethal		—	
2	sublethal		sublethal	
3	sublethal		viable	
4	sublethal		viable	
5	viable		lethal	
6	viable		sublethal	
7	viable		viable	

30 days of age or beyond. The birth event is only a milestone in this continuum. Most deaths occur by about 21 days of age and occur in neonatal (0–5 days) and infant (8–20 days) time period clusters. The distribution of deaths varies a bit among alleles, but the major difference involves *Blo* males. Many of these die with cardiovascular lesions between 50 and 250 days of age, normally a period of minimum death rate.

The prenatal deaths are both preimplantation and postimplantation. For the *To* gene, deaths of *To*

males appear to be equally before and after implantation. No data are now available on the other alleles.

Pathologic Manifestations

The effect of *To* on the elastic laminae of the aorta has been previously described.^(6, 7) Briefly, the elastic laminae are irregular and the intralaminar space appears increased along with disruption of the laminae. Aneurysm, rupture, and hemorrhage are seen. Sometimes aneurysm is accompanied by a defined S-curve in the thoracic aorta or an S-curve may be seen alone. A summary of the incidence of aortic lesions is given in Table 69.

Curiously, the most viable allele, *Blo*, and the most lethal, *To*, have similar frequencies of aortic lesions. Although many *Blo* males die of ruptured aneurysms,

TABLE 69. FREQUENCY OF VASCULAR LESIONS BY GENOTYPE IN THE MOTTLED SERIES

Genotype	N	Normal %	% Aortic lesion	
			Aneurysm	S-curve
<i>Blo</i> +	19	63	32	5
<i>Blo</i> / <i>Blo</i>	13	15	85	-
<i>Blo</i> Y	63	2	98	-
<i>Blo</i> / <i>Mo^{br}</i>	12	100	-	-
<i>Mo^{br}</i> +	32	100	-	-
<i>Mo^{br}</i> Y	13	100	-	-
<i>Mo^{dp}</i> /+	47	90	1	6
<i>To</i> +	328	58	25	17
<i>To</i> Y	26	19	81	-

as have been seen in stillborn *To* males this lesion is obviously not the primary cause of the lethal action. The vascular lesions have not yet been found in *Mo^{br}* or *Blo*, *Mo^{br}*, and the incidence is low in *Mo^{dp}*, yet the lethal action is clearly similar in time to that in *To*. This distinct difference in vascular lesions encouraged our tests for possible recombination between *Blo* and *Mo^{br}*.

One additional feature concerning the possible genetic basis of mosaic pattern variation is discussed in a separate report.⁽⁸⁾

CONCLUSIONS

The genetic and pathologic analysis of the mottled alleles on the sex chromosome of the mouse reveals the complex nature of this genetic locus. The alleles vary in prenatal and postnatal viability, but not in direct relation to the severity of grossly observable lesions of the aorta. The clarity of mosaic coat color patterns also varies among the alleles from distinct patches of depigmentation in both hair and skin of the ears and tail (*Mo^{dp}*) to barely discernible blotches of light hair (*Blo*).

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TENTATIVE LOCATION OF AN X-INACTIVATION CONTROLLER GENE ON THE NORMAL X-CHROMOSOME OF THE MOUSE

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PURPOSE AND METHODS

In the mammalian female, only one of the two X-chromosomes is considered to be functional in the cells and tissues of the adult individual; the other X is heteropyknotic and believed to be metabolically inactive.⁽¹⁾ The choice of which chromosome will be inactive is apparently a random one, and it occurs early in development, probably at or before the definition of the notochord and neural crest.

Melanoblasts are derived from neural crest cells and these migrate laterally to their ultimate location in the skin and hair follicles.⁽²⁾ If one of the X-chromosomes carries a gene that inhibits pigment formation, then an unpigmented area will appear in the coat in those regions where the mutant-bearing X is active. When this X is inactive, the normal coat color will prevail. One allelic series on the murine X-chromosome, the "mottled" series, has this type

of phenotypic expression in the fur.^(2, 3) Females carrying any one of the genes blotchy (*Blo*), brindled (*Mo^{br}*), dappled (*Mo^{dp}*), or tortoise (*To*) have a mosaic coat color. The pattern often shows medial-lateral striation that reflects the migration of melanoblasts.

It is expected that if the choice of inactivation is strictly random, then the average mouse would be 50% greyish-white and 50% full color. Variation around the mean would be essentially binomial and the value of *n* in the expansion to approximate the observed variance would be an estimate of the melanoblast cell population size at the time of X-inactivation.

There is, however, a nonrandom component. The extent of mutant gene expression can be shifted to high or low degrees of expression through ordinary genetic selection procedures.⁽⁴⁾ Lines differentiated by

selection have light coat color areas that make up about 55% and 25% of the total coat for high and low selected lines, respectively. This nonrandom component has been described by Cattanaeh as attributed to an inactivation "controlling element" ⁽⁵⁾ and by Russell as an inactivation center from which inactivation spreads distally. ⁽⁶⁾ To date, all critical published data have been based upon the variegation or mosaicism seen in X-autosomal translocation stocks, wherein inactivation of the autosomal segment does seem to be subject to a spreading effect, or at least to some degree of variation in inactivation not explainable by simple binomial variation.

While this interpretation is certainly valid for the stocks used, its validity concerning the normal X can be questioned. ⁽⁷⁾ Ultimately, it comes down to the question of whether the inactive X is completely inactive or whether it might have a low level of genetic action, a "leaky" effect, that would only be detected in those metabolic systems that have a low threshold of responsiveness. A controlling gene could act to increase or decrease this "leaky" action of the chromosome upon which it is borne. Thus, if a controller that enhances activity is on the X carrying the mottled mutant, the mutant gene effect will predominate so that the average mouse will have in addition to the usual 50% light areas an additional amount due to this low level of gene action from the incompletely inactive X. Conversely, when an enhancing controller gene is on the wild-type X, then nonmutant areas will be added to the expected 50% value, as now the wild-type allele would be irregularly expressing itself in otherwise mutant areas.

This type of gene interaction requires that a type of codominance prevails among the alleles involved. Although strictly speaking, codominance requires both gene effects to be manifest, the interaction we describe is not compatible with partial or complete dominance, either. In this case, we assume that codominance exists to the extent that the gene products of each of the two competing alleles are produced independently of the action of the other X, and the choice of which gene action prevails is random when the phenotypic response threshold has been reached or exceeded.

We, therefore, do not assume that the controlling gene is an "inactivation center" from which a complete inactivating effect will spread with varying extent along the chromosome. Our hypothesis is that all inactive X-chromosomes have some metabolic activity, and the level of this activity is under the genetic control of the controlling element or gene

(which may be synonymous with the inactivation center).

This hypothesis differs from the aggregate of other concepts, as reviewed by Lyon, ⁽¹⁾ in assuming that a), initial X-chromosome inactivation is random but never truly complete regarding gene activity for all sex-linked genes; b), the nonrandom component of variegation is a function of a controlling gene(s) that exerts its effect on the whole chromosome uniformly; c), sex-linked genes can express degrees of dominance and codominance in the heterozygous female similar to autosomal genes, though modified by the level of controlled suppression of activity of the heteropyknotic (inactive) X; d), phenotypic expressions with low thresholds of response can show a wide range of phenotypic variegation along with some response to selection pressure in the heterozygous female; e), two X-chromosomes must be present to produce a completely normal female in all mammals; deviations from this will always lead to abnormality due to gene activity in the presumed inactive X's; f), mutant heterozygotes can show different degrees of mutant expression—the mutant gene may be expressed in the whole animal, but show variegation in the degree of severity; variegated normal versus mutant, uniformly normal, or uniformly mutant—depending upon dominance and threshold of response.

Sex-chromosome genetic behavior, according to these concepts, is then merely an extension of the usual autosomal chromosome and gene behavior, and does not really stress classical genetic concepts as seriously as considered by Grüneberg. ⁽⁸⁾ Proof of the existence of the above considerations is admittedly difficult and may depend mostly upon the consistency of observed phenotypic details and their interpretation. Data of our own on a presumed three-point linkage involving *Mo^b*, *Gs*, and a controlling gene are briefly presented as evidence in partial support of our thesis. Obviously, no single genetic test system could evaluate all the concepts. Provisionally, we will code the controlling gene as *Gg*; the upper-case symbol indicates enhancement of gene activity in the heteropyknotic X.

The data are derived from the F₂ progeny of our present sex-linked mutation rate study. This test employs the + *Mo^b*, + + dam and *Gs* + Y sire to produce an F₁ + *Mo^b* *Gs* + that is outcrossed to CBA males that would be classed as + + Y. Extreme variation in the variegation of brindled, greasy, and brindled-greasy recombinants has permitted a reasonable dichotomization of the brindled and greasy classes that retrospectively suggest that the crosses are as follows:

P	$\frac{Cg + Mo^{br}}{Cg + +}$	$\times$	$\frac{+ Gs +}{+ + +}$	
F ₁	$\frac{Cg + Mo^{br}}{+ Gs +}$	$\times$	$\frac{+ + +}{+ + +}$	
F ₂ ()	$\frac{Cg + Mo^{br}}{+ + +}$		$\frac{Cg + +}{+ + +}$	
	$\frac{+ Gs +}{+ + +}$		$\frac{+ Gs Mo^{br}}{+ + +}$	(17)
	$\frac{Cg Gs +}{+ + +}$		$\frac{Cg Gs Mo^{br}}{+ + +}$	(1)
	$\frac{+ + Mo^{br}}{+ + +}$		$\frac{+ + +}{+ + +}$	(17)

The frequency of phenotypic classes involving Mo^{br} are given in parentheses. These figures suggest about a 5% recombination for each of the regions. The different greasy classes have been more difficult to define, but quite recently we have attempted this classification in mice that are several months old rather than making the determinations at 20 to 30 days as for the other types. At present we detect a class of greasy females that would meet the genotype, $Cg Gs +/+ +$. The patches of Gs on these females are distinctly larger and cover more total coat area than for a female we would class as $+ Gs +/+ +$. The ratio of these two types is at present 4:83.

Probably the most distinctive feature among these mice is the persistent occurrence of minimum Mo^{br} variegation in the $Gs - Mo^{br}$ recombinants—they are a categorically dark class. The one presumed double recombinant is also distinctive and was discernible before weaning as a very light colored, greasy-brindled mouse.

Data collection is continuing, and progeny testing of the F_2 segregants is also under way. The basic

genetic test is also being reconstituted, separately from the mutation rate study, with the original stock presumed to carry the Cg gene.

CONCLUSIONS

The genetic control of mammalian sex-chromosome inactivation is discussed with the suggested hypothesis that the inactive X is never fully inactive and the level of residual activity may be under definable genetic control. Some of the consequences of our thesis are discussed and preliminary data from a three-point linkage are presented to partially support our contentions. Although different interpretations can certainly be applied to our data as they stand, the genetic behavior of the Cg gene can be tested in combination with other sex-linked genes. The hypothesis presented here provides a genetically consistent interpretation of most available data and is, itself, susceptible to genetic test.

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INABILITY TO INHIBIT BRAIN CATALASE COMPLETELY IN VIVO WITH AMINOTRIAZOLE

Robert N. Feinstein, Judith B. Howard, and Joann T. Faulhaber

PURPOSE AND METHODS

Hill et al.⁽¹⁾ recently demonstrated a considerable variation in the sensitivity of various inbred strains of mice to hyperbaric oxygen. These authors were unable, however, to identify the biochemical parameter(s) responsible for the variation, although they did conclude that deaths were due to central nervous

system failure. The enzyme, catalase, seemed to us to be a rather likely candidate for the responsible parameter because of its involvement in oxygen-peroxide metabolism. Two approaches appeared promising to test this possibility: a) obtain number of inbred mouse strains, measure brain catalase, and try to correlate the results with the lethal-

ity data of Hill et al.⁽¹⁾ or b) inhibit brain catalase with 3-amino-1,2,4-triazole (AT) and measure the resistance to hyperbaric oxygen of animals so treated.

The decision to use the latter approach was based on three considerations: a) we would be able to proceed immediately; b) we were, in any case, interested in comparing the effect of AT on brain catalase with its effect on the catalase of other tissues; and c) most importantly, if it developed that low brain catalase was protective against the toxicity of hyperbaric oxygen, we would have available a *tested* technique for the reduction of the catalatic activity of brain. Before measuring the resistance of AT treated mice to hyperbaric oxygen, it was necessary to assure that AT was indeed capable of inhibiting brain catalase *in vivo*, and it is to this question that these experiments were addressed.

Mice used were CF $\times$ 1 Anl[Anl 66] of age and sex specified in each experiment. All injections were intraperitoneal; doses are stated with each experiment. Animals were sacrificed by breaking the neck. The brain (and sometimes also other organs for comparison) was removed and frozen in dry ice-acetone. Frozen tissues were stored over liquid nitrogen and eventually thawed, homogenized in cold 1% Triton,⁽²⁾ and assayed for catalase activity by the perborate technique.⁽³⁾

Some experiments were performed in which AT inhibition of catalase activity *in vitro* was measured. In these experiments, AT effects were measured both in the presence and the absence of ascorbic acid. The ascorbate was added in order to assure the traces of H_2O_2 necessary for AT inhibition.⁽⁴⁾

PROGRESS REPORT

In Vitro Inhibition Experiments

Three experiments were performed with normal brain homogenates, using liver and heart homogenates as comparison tissues. Time and AT concentration were varied, and the effect of ascorbate was tested. In brief, we have found that brain and heart are inhibited *in vitro* by AT as completely as, and more rapidly than, the more thoroughly studied liver. The extent of ultimate inhibition of all tissues was, as commonly found for liver, 92 to 98%. It is further to be noted that although the presence of ascorbate slightly accelerated the inhibition of liver homogenates, it was without positive effect on the inhibition of heart or brain homogenates. Actually, maximal AT inhibition of brain homogenates was slightly delayed by the presence of ascorbate; we have no explanation of this observation. It is, in any case,

evident that there is nothing about brain or heart catalase that makes them inherently noninhibitable by AT.

In Vivo Inhibition Experiments

1. Adult males were given a single injection of 2000 mg AT/kg body weight, then sacrificed at various times, and tissues assayed for catalase activity. Maximal inhibitions attained at any time were liver and kidney, 95%; stomach, 80%; heart, 70%; and brain, 50%. Maximal inhibition was generally observed at about 1 hr after injection.

2. Adult males were given daily injections of 2000 mg AT/kg for 5 days, and groups were sacrificed 1 hr after each injection. Inhibitions observed were: liver, 90 to 95%; heart, 60 to 70%; and brain, 40 to 50%. Only a minor tendency to cumulative, increasing inhibition was observed.

3. Adult males received 2000 mg AT/kg at 0, 6, and 12 hr, and groups were sacrificed at 1, 7, and 13 hr. Inhibitions observed were: liver, 90 to 95%; heart, 65 to 70%; and brain, 40 to 60%. The brain appeared to be accumulating inhibition, but there was so much variation that this cannot be considered significant.

4. Adult males received 2000 mg AT/kg at 0, 2, 4, 6, 8, 10, 12, and 24 hr, and groups were sacrificed 1 hr after each injection. Inhibitions observed were: heart, 70 to 80%; brain, 30 to 60%. Cumulative inhibition was not clearly evident.

5. Because of the possibility that a "blood-brain barrier" is not established in young animals, 14-day-old females were given 2000 mg AT/kg, and blood, liver, and brain were assayed 1 hr later. Inhibitions found were: blood, 0% (AT is known not to inhibit blood catalase *in vitro* in adult animals); liver, 95%; and brain, 67%. Because this was the greatest inhibition observed of brain catalase activity after either single or multiple injections of aminotriazole, it is suggested that the blood-brain barrier may indeed play a role in the control of AT reaching the brain.

CONCLUSIONS

Although *in vitro* tests demonstrate that 3-amino-1,2,4-triazole (AT) can inhibit the catalase activity of heart or brain as effectively (ca. 95%) as that of liver, it has not been possible to achieve as great an inhibition of the brain or heart catalase *in vivo*. Although the blood-brain barrier probably plays a role in guarding brain catalase against AT inhibition, this cannot be the whole story, because stomach and heart catalases are also less inhibited *in vivo*

than are the liver and kidney enzymes. However, brain is inhibited least of all.

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ELECTROFOCUSING OF PURIFIED BEEF LIVER CATALASE AND OF CATALASE IN MOUSE BLOOD

Robert N. Feinstein and Carl Peraino

PURPOSE AND METHODS

Although catalase has been crystallized from blood and liver of several of the larger mammals, and from rodent liver, it has not yet been greatly purified from the blood of either normal or genetically hypocatalasemic mice.⁽¹⁾ This is presumably due largely to the lability of normal mouse blood catalase, which lability is even greater in the case of the hypocatalasemic mutant.⁽²⁾ LKB Produkter (Stockholm, Sweden) has recently developed a series of ampholyte compounds for electrofocusing, and equipment and techniques for their use, with which it should theoretically be possible to fractionate protein mixtures fairly quickly and cleanly on the basis of individual isoelectric points. The initial purpose of this project was an attempt to separate catalase from mouse blood rapidly and with a minimum of degradation. Preliminary results were so unexpected that we have enlarged the problem to include a study of the electrofocusing of purified beef liver catalase. When our understanding of the electrofocusing technique, and of the demonstrated polymorphism of catalase, are more complete, we expect to return to the original attempt at a quick separation of catalase from blood.

Apparatus used was the commercial LKB equipment, and ampholytes of various pH ranges. The ampholyte, with test material incorporated, was placed on a water cooled column in accord with manufacturer's instructions; electric current was applied; and approximately 30 hr later the column was eluted into a standard refrigerated fraction collector. Generally, 50 2-ml fractions were collected. A portion of each fraction was assayed for catalase activity by the perborate technique,⁽³⁾ and the pH of each fraction was individually determined.

Materials that have been examined are beef liver catalase (Worthington Biochemical Corporation,

CTS), and saponin-lysed hemolysates of packed erythrocytes obtained from normal mice.

PROGRESS REPORT

Our first well-defined experiment with mouse blood produced two surprises: a) we found not one, but two peaks of catalase activity, and b) the isoelectric point of both peaks was unexpectedly high, namely, pH 8.1 and 8.9. (The literature⁽⁴⁾ gives an isoelectric point for beef liver catalase of 5.7; we are not aware of any statement regarding the pI of any blood catalase preparation.)

Because of these findings, we decided to confirm the validity of our techniques with a purified beef liver catalase (Worthington, CTS). We have now performed some eight electrofocusing experiments with this material and almost invariably have detected three distinct fractions of active catalase. The ranges of isoelectric points are: Peak I, pH 5.6 to 6.0; Peak II, pH 6.6 to 6.8; and Peak III, pH 7.2 to 7.4. Peak III generally represents only a very small fraction of the total and, in fact, does not always appear. Peaks I and II contain the bulk of the catalatic activity, but the relative proportions in the two peaks vary from experiment to experiment. We have tested the suggestion of Heidrich^(5,6) of "oxidizing" catalase with oxygen and "reducing" it with Cleland's reagent (dithiothreitol). Our first experiments suggested that Peak I represented a reduced form of catalase and Peak II an oxidized form, but later experiments have not been consistent on this point. We are still unable to predict the relative proportions of catalase that will appear in the two major peaks.

It should be noted that heterogeneity of liver catalase is not a new observation. Recent mention of this phenomenon will be found in the papers of Heidrich^(5,6) and of Nishimura et al.⁽⁷⁾

CONCLUSION

Isoelectric heterogeneity is shown by both the catalase in whole lysates of mouse erythrocytes, and also by a purified commercial beef liver catalase. The isoelectric points observed for mouse blood catalase are approximately three pH units higher than for beef liver catalase.

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THE GROWTH-DUPLICATION CYCLE. X. APPEARANCE OF PHAGE BINDING SITES DURING THE CELL CYCLE OF ESCHERICHIA COLI

Michael L. Freedman and Herbert E. Kubitschek

PURPOSE AND METHODS

If cells grow linearly because the number of sites for uptake of nutrients remains fixed, then the rate of accumulation of nutrients during most of the cell cycle should be constant. Such a response was found in *Escherichia coli* strains B_s-1, WP2 her⁻, and 15 THU for the metabolites glycine, leucine, glucose, sulfate, phosphate, and thymidine.⁽¹⁾ Because the uptake loci for these substances may lie in the cell membrane, the study has been extended to the bacterial cell wall through the use of isotopically labeled bacteriophage.⁽²⁾ We have examined *E. coli* 15 THU and B r to see whether or not the number of phage attachment sites is constant during most of the cell growth cycle.

Preparation of Radioactive Bacteriophage

We used coliphage T4D, which requires thymidine, and host strains B3 or 15 THU, which are thymidylate synthetase negative. Lysis occurred in supplemented minimal medium with methyl-³H-thymidine as the sole thymidine source. The lysates were extracted with deoxyribonuclease I and dia-

lyzed against basal salts medium to remove tritium counts not associated with phage. The phage were concentrated by acid precipitation, and an aliquot was banded in a cesium chloride gradient using an analytical ultracentrifuge. The results of such a phage purification procedure are shown in Figure 108. Less than 9% of the radioactivity lies outside the phage-containing samples.

Assay of Phage Binding Sites

To follow the appearance of phage adsorption sites during the cell cycle, shake cultures of *E. coli* 15 THU and B r were maintained at cell titers characteristic of steady state growth by repeated back-culture. These growing cultures were exposed to phage at different multiplicities of infection for 3 to 4 min, after which time formalin was added to kill the cells. In a few experiments the cells were concentrated 10- to 15-fold prior to infection. Unadsorbed phage were removed by centrifuging and resuspending in fresh medium. Figure 109 shows the effect of repeated wash cycles at several cell concentrations and phage multiplicities. Once binding has occurred (measured after

FIG. 108.—Distribution of lytic activity and radioactivity in a preparation of coliphage T4D banded in a cesium chloride density gradient. Sample densities diminish toward the right.

FIG. 109.— Comparison of radioactivity bound to cells after several washes following infection of *E. coli* strains with phage T4D. Several cell densities and phage multiplicities of infection (MOI) were used.

FIG. 110. Radioactivity per cell as a function of the ratio of the mean cell size, V_s , to the mean birth size, V_b . *E. coli* strains 15 THU and B/r were infected with T4D phage at several multiplicities of infection, MOI. CV is the average coefficient of variation of the samples taken from the sucrose gradient.

the first wash) little activity is lost. As expected, the lower the cell titer, the smaller is the percentage of the available tritium counts bound. This appears true even when high phage multiplicities were used with low cell concentrations.

Finally, the formalin killed cells with their bound

phage were subjected to velocity centrifugation through a 5 to 15% gradient. Cell samples from the gradient were counted and sized with a Coulter counter-multichannel analyzer, and their radioactivities were measured. Radioactivity per cell was expressed as a function of average cell size.

PROGRESS REPORT

Figure 110 shows the results of five phage-binding experiments. The specific activity of the T4D phage used in experiments shown in Figure 110 e and d was 2.1×10^{-4} cpm phage and in experiments a, b, and c, 0.6×10^{-4} cpm phage. Regardless of the host cell strain or the multiplicity of infection, the phage-associated radioactivity bound per cell remained essentially constant for approximately the first two-thirds of the cell cycle. In Figure 110 b and d, where mean cell sizes, V_s , were larger than 1.6 times the mean birth size, V_b , there was an abrupt increase in the amount of radioactivity bound per cell.

CONCLUSION

From these experiments, it appears that the number of receptor sites for the attachment of bacteriophage T4D in *E. coli* strains 15 THU and B/r remains constant for about two-thirds of the cell growth cycle and then increases abruptly. The results are in good agreement with measurements of the number of uptake sites for metabolites. Also, the results suggest that phage attachment sites are either formed or activated comparatively late in the cell growth cycle.

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THE GROWTH-DUPLICATION CYCLE. XI. GROWTH OF NONDIVIDING CELLS DEPRIVED OF THYMINE

Herbert E. Kubitschek

PURPOSE AND METHODS

Evidence for linear growth of bacteria was presented earlier in this series. Linear growth requires that the rate of increase in cell mass is constant. In turn, this implies that during most of the cell cycle there are a constant number of surface sites for the uptake of each growth factor. Measurements of the rate of uptake of a variety of growth factors support this interpretation.⁽¹⁾

This interpretation predicts that nondividing cells would grow indefinitely at the same rate as long as the number of uptake sites remained constant. It might also be necessary to prevent DNA synthesis to maintain a constant number of sites. Fortunately, both DNA synthesis and cell division are prevented in thymine-requiring bacteria that are starved for this base. These thymine-starved cells become inviable ("thymineless death") if they have not completed a

FIG. 111. —Total and viable counts of a culture of *Escherichia coli* THU deprived of thymidine and of a control culture.

The cultures were grown with acetate as the energy source. Total counts were measured with a Coulter counter. Viable counts were determined by plating cells upon nutrient agar. ○, ●, without thymidine; △, with thymidine.

round of DNA replication. Nevertheless, if an energy source is present, the cells should continue to increase in size, and their rate of increase would then provide a measure of the number of uptake sites.

PROGRESS REPORT

A synchronous culture of *Escherichia coli* THU, requiring thymine (or thymidine), histidine, and uracil, was prepared by banding cells in a sucrose gradient in a centrifuge tube and selecting small cells from the top of the band. The culture was divided into two parts, one of which was deprived of thymidine. As shown in Figure 111 the cells in the control culture divided completely after about 3 hr, and again after about 5 hr. The number of cells in the thymidine-deprived culture did not increase and, in fact, decreased slightly after about 4 hr. The viable count in this culture decreased by a factor of more than 200 during the experiment.

The mean cell volume of the control culture increased linearly before division (Figure 112), and after division was completed (by the third hour) the

FIG. 112. Mean cell volumes for the same cultures as in Figure 111.

growth of the progeny was again linear and had the same rate as the parental generation. In the thymidineless culture the growth rate was reduced temporarily, an unusual occurrence probably due to a handling accident. After this time, mean cell volume in these cells increased at the same rate as in the control culture. The cells deprived of thymidine maintained the same growth rate as the control during the remainder of the experiment, as cell volumes increased through more than two successive doublings.

From microscopic examination, thymineless cells were irregular in shape and length, with pronounced changes in density in different areas. Even though these cells were aberrant and inviable, their growth rate was unaffected, indicating that the number of uptake sites remained constant.

CONCLUSION

Further support for a constant number of uptake sites (for transport of growth factors into the cell) during the cell cycle is provided by the observation that growth rates remain constant for at least two generation times in cells that are prevented from DNA synthesis and cell division by thymine starvation, and that these growth rates are equal to those of cells in the steady state growth-duplication cycle.

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MUTATION IN CONTINUOUS CULTURES: INTRODUCTION

Herbert E. Kubitschek

Continuous culture techniques allow the study of mutation *in vivo* under carefully controlled conditions and present many advantages over batch cultures. Cell death and physiological perturbations are minimized. Effects of toxic mutagens can be examined by maintaining concentrations of these compounds at very low levels. Bacteria in slowly growing cultures contain a minimum amount of DNA, presumably a single genome, desirable for studies of mutation. In addition, accurate data are more easily obtained in continuous cultures than in batch cultures, because the technique provides a transformation from the exponential increase of cell numbers in flasks to the linear increase of cells in continuous cultures. This transformation is both an experimental advantage, providing constant numbers of cells, and a conceptual advantage, allowing the investigator to think more directly about his research problem.

Most of our effort has been devoted to studies of the kinetics of accumulation of mutants that are re-

sistant to bacteriophage T5 in chemostat cultures of *Escherichia coli*. This is a forward mutation with the advantage that this mutant character is not under selection. As a result, there is no need to determine the value of a selection factor for each experiment. Concentrations of phenotypically expressed mutants were measured by exposing culture samples to bacteriophage T5 upon nutrient agar plates; sensitive cells adsorb the bacteriophage and are lysed, while mutant cells do not adsorb the phage and generate visible colonies after a day's growth at 37°C. Concentrations of latent mutants (cells with the wild phenotype, sensitive to T5 and giving rise to mutant descendants resistant to T5) were measured by spreading culture samples upon plates, and incubating these at 37°C for 3 hr before spraying with bacteriophage. During this period, each cell generated a microcolony of about 100 cells, allowing expression of the mutation in one or more of the progeny of a newly mutated cell.

MUTATION IN CONTINUOUS CULTURES. I. REPAIR OF UV-INDUCED LETHAL AND MUTATIONAL LESIONS

Herbert E. Kubitschek

PURPOSE AND METHODS

Although we have observed repair of mutational lesions in discontinuous cultures, present evidence supports the absence of repair of mutational lesions for T5 resistance in continuous, glucose limited chemostat cultures, even in bacterial strains known to produce repair enzymes under other growth conditions.⁽¹⁾ This absence of repair of mutational lesions led us to ask 1) Are lethal lesions repaired in chemostat cultures? and 2) Are lethal lesions basically different from mutational lesions?

To examine the first question, bacteria from chemostat cultures were exposed to ultraviolet light (UV) and plated on chemostat-like media. The survival of these cells was compared with that for the same strain grown in nutrient broth, irradiated, and plated on nutrient agar. The strain used, *Escherichia coli* B, is known to repair lethal lesions when grown on nutrient agar. Thus, if survival of the chemostat cells is equal to that for nutrient-grown cells repair must occur as extensively for both. Minimal plates for the chemo-

stat cells contained glucose at a concentration of 10 µg/ml. This low concentration is approximately that found in chemostat cultures, so the growth of cells on these "chemostat" plates mimics the growth in chemostat cultures.

Alternatively, UV-irradiated bacteriophage T1 was plated with cells under the two growth conditions. Lethal lesions in phage T1 can be repaired by *host cell reactivation*; enzymes produced by the host bacterium repair the lesion in the phage. Again, repair must be as extensive in chemostat cultures as it is on nutrient agar plates if phage survive equally well under both conditions.

To examine the second question, cells from irradiated chemostat cultures were exposed to visible light, which is known to repair pyrimidine dimers that are produced in DNA by UV. If these dimers are the mutational lesions, or if they are the *source* of some of them, exposure to visible light should reduce mutant frequencies. A corresponding experiment, measuring cell survival would examine the possibility that dimers are the source of lethal lesions.

FIG. 113. Survival of *E. coli* B after exposure to ultraviolet light for two different conditions of growth. ○, grown in nutrient broth, irradiated, and plated on nutrient agar; ●, grown in glucose limited chemostat culture, irradiated, and plated on "chemostat plates."

PROGRESS REPORT

Cells of *E. coli* B were grown upon nutrient broth, exposed to UV, and plated upon nutrient agar. The survival curve for this culture was experimentally indistinguishable from cells grown in chemostat cultures and plated upon "chemostat plates," Figure 113. Since cell survival under nutrient conditions involves extensive repair of lethal lesions, the similar survival under conditions of chemostat culture requires that repair processes occur to about the same extent.

In a second comparison, UV-irradiated bacteriophage T1 were mixed with cells of *E. coli* B r grown in nutrient broth or in chemostat cultures, and plated upon corresponding media. Again, survival curves were experimentally indistinguishable, Figure 114. Since extensive host cell reactivation of T1 occurs with these broth-grown cells, repair must also have occurred under chemostat conditions.

To examine the nature of the mutational lesions, a chemostat culture of *E. coli* WP2 *try hcr* was ex-

FIG. 114. Survival of bacteriophage T5 after exposure to ultraviolet light on *E. coli* B/r grown under two different culture conditions. ○, grown in nutrient broth, irradiated, and plated on nutrient agar; ●, grown in glucose limited chemostat culture, irradiated, and plated on "chemostat plates."

FIG. 115.—Cell survival and photoreversal of latent mutants in a chemostat culture of *E. coli* WP2 her exposed to visible light.

FIG. 116. Photoreactivation of cells from a chemostat culture of *E. coli* WP2 her by exposure to visible light after exposure to UV. Visible light exposure (PR) approximately 10^6 erg mm^{-2} .

posed to UV, and a generation later, samples from this culture were exposed to visible light (100-W tungsten projection lamp; Kodak Wratten filter #85 to remove wavelengths below 380 nm; dose rate, ap-

proximately 5×10^6 erg mm^{-2} hr^{-1}). As shown in Figure 115, mutant frequencies were decreased by about 65% at exposures that killed only small fractions of these cells. This decrease provides evidence for the photoreversal of pyrimidine dimers that act as mutational lesions, or as the source of these lesions.

Finally, lethal lesions produced in UV-irradiated cells from chemostat cultures of *E. coli* B r were photoreactivated with visible light, Figure 116.

CONCLUSIONS

After exposure to UV, both lethal lesions and mutational lesions were repaired when chemostat cells were exposed to visible light. Because repair by visible light is thought to involve only pyrimidine dimers, it appears that these dimers are responsible both for death and mutation.

Nevertheless, repair systems for death and mutation must be different, because lethal lesions were repaired in continuous chemostat cultures while mutational lesions to T5 resistance were not. Since the same cells are known to repair mutational lesions under other growth conditions, the corresponding repair system must be inactive in chemostat cultures. This may be due to the limited energy supply in these cultures. If so, there can be no repair of other mutational lesions. This possibility is under investigation.

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MUTATION IN CONTINUOUS CULTURES. II. SPERMINE AND CADAVERINE AS ANTIMUTAGENS

Herbert E. Kubitschek

PURPOSE AND METHODS

Antimutagens reduce spontaneous or induced mutation rates, without reacting or complexing with the mutagen. A knowledge of the antimutagen spectrum for a given mutagen is of importance in determining the mode of action of the mutagen *in vivo*. Spermine was claimed to be antimutagenic by Johnson and Bach,⁽¹⁾ who found that this compound reduced both spontaneous and caffeine induced mutation rates to streptomycin resistance in batch cultures of bacteria and that spermine complexed with the bacterial

DNA.⁽²⁾ Their experiments, however, did not rule out the possibility that streptomycin resistant bacteria had a selective advantage in the presence of spermine. Mutation to T5 resistance in chemostat cultures avoids this problem because T5 resistant bacteria grow at the same rate as their sensitive parental strains.

PROGRESS REPORT

Spermine was found to be antimutagenic for caffeine induced mutations to T5 resistance in glucose

limited chemostat cultures of *E. coli* B/r, 1, try. At a concentration of 40 μ M, spermine decreased mutation rates by a factor of four. The related diamine, cadaverine was less efficient. At a concentration of 110 μ M, cadaverine decreased mutation rates by about a factor of three.

CONCLUSIONS

The diamines, spermine and cadaverine, were found to be antimutagenic against caffeine induced mutation in chemostat cultures. These diamines are believed to complex with DNA as do other antimutagens. If, as Puglisi has suggested,⁽³⁾ antimutagenic

compounds act through inhibition of nucleotide removal of some portion of the DNA, we may expect most compounds that complex with DNA to be antimutagenic.

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MUTATION IN CONTINUOUS CULTURES. III. NUCLEAR SELECTION

Herbert E. Kubitschek

PURPOSE AND METHODS

Cells in tryptophan limited chemostat cultures of *E. coli* B 1, try and B r 1, try are filamentous.⁽¹⁾ At generation times longer than 2 hr, the average volume and DNA content of these multinucleate, filamentous cells is proportional to the generation time of the culture.⁽²⁾ When such cells are exposed briefly to the mutagenic action of acridine orange and visible light, mutant frequencies (to T5 resistance) are increased immediately and then remain almost constant there-

after. This constancy requires the expression of T5 resistance in the progeny of almost every newly induced mutant.

However, T5 resistance is a recessive character, and therefore should not be expressed unless all nuclei are mutant. Because mutation is a very rare event, it is unlikely that more than one nucleus was mutated in any cell. Thus, most of the nuclei in mutated cells should have been wild type, and consequently, most progeny cells were expected to be unmutated. Instead, most or all of the progeny of newly mutated cells ultimately were observed to express the mutant character.

The length of these filamentous cells and the number of nuclei they contain both depend upon generation time. These filamentous cells can be fractured to individual cells, each containing a single replicating nucleus by decreasing the generation time of the culture. When nuclei are "repackaged" into individual cells in this way, the phenotype of the nucleus is expressed. Thus, a repackaging experiment (by a shift to more rapid growth rate) makes it possible to determine the number of mutant nuclei originally present in the filamentous cells.

Four possible models of nuclear replication and segregation are illustrated in Figure 117, for cells containing four nuclei. These are:

FIG. 117. Models for mutation, selection, and segregation of nuclei in filamentous bacteria.

A. All nuclei replicate at the same rate and produce daughter nuclei that remain adjacent to each other. As shown in the figure, this model requires expression of progeny in 25% of these multinucleate cells. Since final mutant frequencies were almost equal to frequencies initially induced, this model is ruled out immediately. The other three models are consistent

FIG. 118. Predictions of mutant frequencies after "repackaging" nuclei into individual cells.

with complete expression of mutation in all progeny of newly mutated cells.

B. All nuclei in a cell are mutated by a single mutational event.

C. Resistance to bacteriophage T5 is a dominant character in these cultures, and pairs of mutant nuclei are regularly segregated.

D. Only the mutated nucleus is replicated and transcribed.

Models B, C, and D differ in their predictions of mutant frequencies for repackaging experiments immediately after mutagenesis and at much later times. These predictions are illustrated in Figure 118.

PROGRESS REPORT

A slowly growing culture with a generation time of 8.3 hr was exposed to acridine orange and visible light to produce mutants for the repackaging experiment. When cells were taken from this culture immediately after mutation and shifted to a generation time of 2.1 hr, the mutant frequency dropped to a much lower steady state value, as shown in Figure 119. This result rules out model B, the mutation of all nuclei.

When cells were taken from this culture after 6.2 generation times, mutant frequencies were unchanged. This shows that almost all nuclei in the filamentous progeny of mutant cells were mutant, ruling out model C. The only model that explains these results, therefore, is the final model, D, of a "master nu-

FIG. 119. A "repackaging" experiment performed immediately after brief exposure of a glucose limited culture of *E. coli B r 1, try* to the mutagenic effect of acridine orange and visible light.

cleus": only one nucleus in the cell that is transcribed and replicated. Master nuclei of subsequent generations are the descendants of the original master nucleus.

CONCLUSIONS

The filamentous bacteria formed in tryptophan limited cultures of the tryptophan requiring strains of *E. coli B 1, try* and *B R 1, try* have only a single functional, or "master" nucleus. All other nuclei are dormant. The phenotype of the cell is determined by the genetic constitution of this nucleus, even for recessive characters. The master nucleus is the progenitor of all nuclei formed in these cells, including the master nuclei of successive generations. This extreme selection probably requires that replication or initiation of replication of DNA is limited by the supply of the required amino acid under these growth conditions.

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MUTATION IN CONTINUOUS CULTURES. IV. DELAYED EXPRESSION OF MUTATION TO T5 RESISTANCE

Herbert E. Kubitschek

PURPOSE AND METHODS

Expression of mutation cannot occur instantaneously. Mutant proteins or enzymes must be made, or wild-type enzymes must be diluted out by growth and division. If the character is recessive, nuclear segregation may be required for expression. Furthermore, mutation may involve only a single strand of the DNA duplex, and transcription and expression may be delayed until the mutant locus is replicated to the second strand. These, and other factors, may add to the period of time required for expression of the mutant phenotype.

Delay periods found for expression of azide resistance⁽¹⁾ and for reversion to independence of amino acids⁽²⁾ generally have been rather short, about a generation time or less. Far longer delays occur in the expression of resistance to bacteriophage T5 and other phages, with mean delay periods that approach five generation times.⁽³⁾ The long delay in the expression of resistance to T5 and other bacteriophages has been a perplexing problem for over a decade.

Sensitivity to bacteriophage T5 is a dominant character, and it is due to the presence of active receptor sites on the bacterial surface. Working with phage T5, Weidel and his coworkers extracted active receptor sites from bacteria. The extracted sites were mucopolysaccharide spheroids, and a single phage could attach to each.⁽⁴⁾ Receptor sites extracted from resistant bacteria had the same chemical, morphological, and immunological properties, but phage were not able to attach.^(5, 6)

An early proposal for the long delay period for phage resistance was that, after mutation, functional receptor sites were diluted out to progeny cells by growth and division. Ultimately, some progeny would arise that had inherited no active sites, and therefore would be sensitive to the phage. Although this model was attractive, it is ruled out by the finding that the delay period for T5 resistance depends upon the mutagen to which the cells are exposed: with caffeine the mean delay period is almost 5 generations, but with acridine orange and visible light as the mutagen, the delay is only half as long.⁽⁷⁾ The same number of receptor sites are present on these cells before either mutagen is added, and the same number of generations would be required in each case to dilute them out. In addition, the mean delay of 2.5 generations

for acridine orange also rules out the site-dilution model, since the average number of receptor sites per cell could not be greater than 10, if these are to be diluted away in 2.5 generations. On the contrary, measurements of the number of receptor sites always give much larger numbers, from 200 to 1000 per cell.

It is interesting that other mutagens also appear to give delays that fall into one or the other of the two classes. For example, similar long mean delay periods were found for visible light mutagenesis, 4.4 generation times (R. B. Webb of this Division) and for rapidly growing cultures exposed to UV or to 2-aminopurine, approximately 5 generations. Shorter delays of approximately 2.5 generation times were observed with visible light in conjunction with any of several other dyes, proflavine, 5-aminoacridine, methylene blue, and also in very slowly growing chemostat cultures exposed to UV or 2-aminopurine.

Because the cells in these cultures were uninucleate, these results are not explained by nuclear segregation, nor are they explained by any simple models of additive delay.

PROGRESS REPORT

Models to describe the observed delays in phenotypic expression of T5 resistance were constructed on the basis of results reported earlier for acridine orange visible light as the mutagen.⁽⁸⁾ At each division, a latent mutant cell gives rise to one daughter that remains sensitive to the phage and one daughter that expresses resistance. Schematically,

where *L* represents a latent mutant cell and *E* represents a cell that has expressed the mutant phenotype. The mean delay for this sequence is 2 generations. In addition, assuming that mutation only occurs or is fixed at replication of the gene, the average period before the gene is replicated in cells distributed throughout the cell cycle in continuous cultures is approximately 0.5 generations. Thus the value of the mean delay period for this model is approximately 2.5 generations in continuous cultures. This value is in excellent agreement with the short delay values observed experimentally.

A second model assumes that a newly formed latent mutant (L_1) must go through a second latent step (L_2) before the mutation can be expressed (E). Schematically,

The mean delay period of this sequence is 4 generations. Adding the period of 0.5 generations for cells in continuous cultures, the mean delay period for this model is 4.5 generations. This value is in good agreement with the longer delay values observed experimentally.

Neither of these models for phenotypic expression agrees with the conventional Watson-Crick model for replication of DNA, because all the progeny of each latent mutant give rise to expressed mutants. Both schema predict absence of segregation after mutation, and are in agreement with observed results,⁽⁸⁻⁹⁾ quantitatively as well as qualitatively. These delay models support the master strand model of DNA replication proposed earlier.⁽¹⁰⁾

CONCLUSIONS

Two models were proposed for expression of mutation to T5 resistance. The delay periods predicted by these models are in agreement with experimental results. Both of the delay models require that DNA

replicate from a master strand, and not from both strands as in the Watson-Crick model.

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CHANGES IN THE MEAN CELL VOLUME OF ESCHERICHIA COLI AFTER BACTERIOPHAGE INFECTION

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PURPOSE AND METHODS

There have been a very limited number of studies of the effect of bacteriophage infection on bacterial cell size. Using motion photomicrography, Bayne-Jones and Sandholzer⁽¹⁾ (1933) showed an apparent swelling of individual *Bacterium coli* cells which had been exposed to lytic principle. Cell sizes increased as much as 900% prior to lysis when compared to uninfected controls of similar age. In 1948, Doermann⁽²⁾ reported a decrease in turbidity after infection of *Escherichia coli* by wild type and by rapid lysis mutants of phages T2, T4, and T6. The recovery of the culture from the drop in turbidity was slower at higher multiplicities of infection (MOI). More recently, indirect evidence has appeared that suggests physical swelling of phage infected *E. coli* cells. Con-

comitant with infection and dependent on phage multiplicity, ion fluxes, optical density decreases, and packed cell volume increases occur in *E. coli* K12 (λ) infected with T4rII mutants and in *E. coli* B infected with T-even phage.^(3,4)

In the present study, we have used a Coulter counter-multichannel analyzer to investigate directly the response of host cell size to phage infection. Changes in cell number and mean cell volume were followed for cultures of *E. coli* B r and B_{r-1} infected with phage T1 or T2r. Shake cultures in Cas-amino acids-supplemented basal salts medium were maintained by repeated back-culture at low cell titers characteristic of balanced growth. Bacteriophage were added at MOIs from <0.1 to >520. Cell aliquots from infected and from uninfected control cultures were re-

FIG. 120—Cell volume distributions of control and phage infected cultures of *E. coli* B'r. V. culture infected with viable phage; UV, culture infected with ultraviolet killed phage. Multiplicity of phage T2r was 4.0. Arrows denote mean cell sizes.

moved every few minutes, and both titered and sized electronically. Both virulent and ultraviolet light (UV) killed phage were used in most experiments.

PROGRESS REPORT

The results from a typical experiment are shown in Figures 120 and 121. Figure 120 presents the cell volume distributions from control and phage infected cultures and the volume distribution of 1.305- μ diameter standard microspheres. A marked shift to a larger mean cell volume is seen with time in cultures infected with either viable or with UV killed phage. Figure 121a gives the change in Coulter counts (corrected to true cell counts/ml) and reveals, characteristically, a slight increase in cell number in both infected cultures. (The control curve translates to a straight line on a semilogarithmic plot.) The mean cell volumes in Figure 121b reveal steady state constancy for the control and increases for the experimental groups. The total volume of cellular material per milliliter of culture, Figure 121c, is obtained from the product of Figures 121a and b. It shows an immediate deviation from exponential increase in the infected cultures.

The dependence of swelling upon MOI is presented

in Figure 122. The percent increase in the mean cell volume, after 20 min, of *E. coli* infected with viable phage was arbitrarily selected as the basis for comparison. At MOI > 4, the cells' ability to swell reaches a plateau. Figure 122 indicates that swelling is not influenced by the phenomenon of "lysis from without," which has been described for high titers of phage.

A possible explanation for the above results is that phage infection immediately blocked cell division without inhibiting the increase in cell volume and mass associated with normal cell growth, i.e., that phage infection permitted cell growth without division. If this were the case, the mean dry weight per cell should increase with time after phage infection. In order to test this possibility, large aliquots of infected cultures were trapped on membrane filters, washed, and dried in vacuo at 80°C. The results of such an experiment are presented in Figure 123. The mean dry weight per cell remains constant, as anticipated if the size increase were due to physical enlargement of the infected cell without concomitant

FIG. 121.—a, Plots of cell number increase; b, mean cell volume changes; and c, total cell volume changes per milliliter of culture. Same cell, phage, and MOI as in Figure 120.

FIG. 122. Dependence of increase in mean cell volume after 20 min of infection. Data are combined from experiments using *E. coli* B₁ and B_r and phages T1 and T2.

FIG. 123. a. Constancy of mean cell dry weight; and b, increase in mean cell volume of *E. coli* B₁ infected with viable or UV-killed T2r phage at an MOI of 2.6.

macromolecular synthesis. The abrupt increase in the mean cell volume, 45 min after infection with viable phage, is thought to reflect lysis of the bacterial culture, which begins about 30 min after infection. Lysis could cause this increase in the mean dry weight per cell, because the cell titer drops considerably, while much of the resulting debris is retained on the filter and contributes to the total dry weight. Ultraviolet light killed phage would not be expected to cause lysis to the same extent as virulent phage.

CONCLUSION

Swelling of the bacterial host cell appears to be a real and significant stage in the infective cycle of *E. coli* bacteriophage. It occurs early during the infective cycle, does not seem to be reversible, requires very low multiplicities of infection, and does not, in fact, depend on the action of a viable phage. It does not reflect any increase in dry weight per cell. These results are all compatible with the recent suggestion of S. S. Cohen⁽⁵⁾ that such swelling is an osmotic phenomenon caused by the phage's puncturing the bacterial cell wall and membrane. Our present data do not distinguish whether or not this swelling plays a vital role in the phage life cycle as postulated by Cohen. Further experiments are planned along this line.

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EFFECTS OF CELL SIZE AND DNA SYNTHESIS ON RADIATION SENSITIVITY OF *ESCHERICHIA COLI*

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PURPOSE AND METHODS

Studies of ultraviolet sensitivity in synchronously dividing populations of *Escherichia coli* have demonstrated differences among cells of different ages after division.^(1,2) In these experiments cells with relatively short generation times, continuous DNA synthesis, and varying numbers of nuclei per cell were used. The studies demonstrated an increase in the number of survivors in the latter part of the cell cycle, identified by an increase in the initial shoulder of the survival curve but with no change in its final slope.

The goal of the experiments presented here was to determine correlations between radiation sensitivity and either cell size or DNA synthesis in unsynchronized, slowly growing, largely uninucleate cell populations, obtained by growing *E. coli* in steady state chemostat cultures. Growth was limited with 100 $\mu\text{g}/\text{ml}$ glucose, and the generation time was varied from 2 to 12 hr by regulating the rate at which fresh medium was introduced. Cell samples with different mean volumes were prepared by velocity sedimentation of the entire cell population in a sucrose gradient.⁽³⁾ Cell volume distributions for each sample were determined in a Coulter counter coupled to a multi-channel analyzer.

These techniques allow the study of differences in radiation sensitivity among cell populations of different mean volumes. Because the size of individual cells of *E. coli* increases with their age after division, these techniques also permit the study of differences in radiation sensitivity among cell populations of different mean ages. Experimental results obtained from unsynchronized cell populations thus can be compared directly with results obtained from mechanically or chemically synchronized cell populations.

It had been shown previously that DNA synthesis in slowly growing chemostat cultures of *E. coli* 15 THU is restricted to the larger cells at generation times greater than 2 hr.⁽³⁾ Radiation sensitivity in either the presence or absence of DNA synthesis is studied by irradiating samples containing cell populations of mean volumes both above and below the critical value at the onset of DNA synthesis.

PROGRESS REPORT

Experiments were carried out with both *E. coli* B_{s-1}, which is deficient in dark repair and is very UV

sensitive, and *E. coli* 15 THU, which has normal dark repair and is UV resistant. The bacteria had generation times of 6 to 7½ hr, insuring a single nucleus at birth and the absence of DNA synthesis until late in the cell cycle. Cell samples of four different mean volumes were used in each experiment. In a single experiment, the mean cell volume for the sample with the largest cells was, on the average, about 65% greater than the mean cell volume of the sample with the smallest cells. Survival curves were obtained for these cell fractions by irradiating them with a range of UV doses.

Within experimental errors, the survival curves for repair deficient *E. coli* B_{s-1} cells of different sizes were the same. On the other hand, small, but significant, differences were obtained with *E. coli* 15 THU cells of different sizes; the larger cells had more survivors at low doses. Furthermore, UV survival curves for *E. coli* B_{s-1} were exponential, but those for *E. coli* 15 THU had an initial shoulder. For both strains, however, an analysis of the data using least squares fits indicated that the final slopes of the survival curves were independent of mean cell size.

CONCLUSION

Cell size, age, and the presence or absence of DNA synthesis have no apparent effect on the UV survival curves *E. coli* B_{s-1} (a repair deficient strain), nor upon the final slope of *E. coli* 15 THU (a strain capable of repair). The magnitude of the initial shoulder on the survival curve of 15 THU, which has a dark repair mechanism, increases with cell volume.

The underlying mechanism for the increased resistance of 15 THU is not identified. Because it is seen only at low UV doses in cells with a repair capacity, we suppose that this differential sensitivity may reflect greater repair capacity in larger cells, possibly due to a greater number of repair enzymes in these cells. Our results are in general agreement with the previously cited reports of differential UV sensitivity in synchronously dividing bacterial populations that have normal repair capability.

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LETHAL AND GENETIC EFFECTS OF RADIOISOTOPE DECAY IN BACTERIOPHAGES AND BACTERIA

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PURPOSE AND METHODS

Various radioisotopes incorporated into viable cells cause death or genetic damage in an increasing fraction of the cells as decay occurs. These effects have been most extensively studied for ^3H and ^{32}P .^(1, 2) It is generally difficult to relate observed biological effects in such experiments to specific physical and chemical changes in decaying atoms.⁽³⁻⁵⁾ In fact, it often is impossible even to distinguish effects due to these local changes in the decaying atom from those due to irradiation of the rest of the cell.

In the study reported here, the effects of radioisotope decay in bacteriophage are investigated and compared with the effects of decay in bacteria. By using bacteriophage, the smallest available microorganism, it is hoped that the role of radiation effects in comparison to local decay effects can be minimized. Studies of the lethal and genetic effects of the beta emitters ^{32}P , ^{33}P , and ^{14}C , as well as ^{125}I , which decays by electron capture, are included. Each isotope is incorporated into the DNA of the microorganisms to be studied.

^{32}P and ^{33}P emit beta particles with mean energies of 0.70 MeV and 0.093 MeV, respectively, and mean ranges in water of 2600 μ and 120 μ ; and the daughter nuclei acquire mean recoil energies of 20 eV and 1.7 eV. Decay schemes otherwise are identical. It has been proposed that the lethal effects of ^{32}P in cells are primarily due to double-strand breaks in DNA caused by the high recoil energy.⁽¹⁾ If this were true, the lethal effects of ^{33}P decay would be much less than those of ^{32}P decay. Only two experiments are reported that tested this idea, and their results are contradictory.⁽¹⁾ By incorporating purified ^{32}P or ^{33}P into bacteria and bacteriophage and comparing lethal efficiencies, it is proposed to test this hypothesis regarding the lethal mechanism of ^{32}P decay.

^{14}C decays by emitting a beta particle of relatively low energy (mean 0.05 MeV). Its lethal effects have been studied by others using bacteria and various higher organisms.^(4, 5) With our present test system, the difficulty of distinguishing between local decay effects and radiation effects should be greatly de-

creased because of the small size of the T1 phage particle (0.05 μ) compared with the mean range of ^{14}C beta particles in water (33 μ).

^{125}I decays by electron capture, with a subsequent vacancy cascade and probable electrostatic disruption of the molecule harboring the decaying atom.⁽⁶⁾ ^{125}I is being incorporated into phage DNA in the form of labeled iododeoxyuridine, a thymidine analog. Although the biological effects of ^{125}I decay have not been studied, the disruptive local physical events known to accompany its decay lead to the expectation of a high lethal efficiency for decays in DNA, perhaps 100%.

PROGRESS REPORT

Experiments have been undertaken to compare the effects of ^{32}P and ^{33}P decay in bacteriophage T₁ and in several strains of *E. coli*: two wild-type radiation resistant strains, 15 THU and K-12 AB 1157 as well as two mutant radiation sensitive strains, B_{s-1}, which is deficient in dark repair of DNA damage, and K-12 AB 2463, which is recombination deficient. Preliminary results indicate that the lethal effects of ^{33}P duplicate those of ^{32}P in the phage and in the four bacterial strains. When storage during decay is at -196° C, approximately 5% of all decays in DNA are lethal to phage T₁ or to either radiation sensitive strain of *E. coli*, whether the isotope is ^{32}P or ^{33}P . Approximately 2% of ^{32}P or ^{33}P decays in DNA are lethal to either of the radiation resistant strains of *E. coli*.

In other experiments, the lethal effects of ^{125}I decay in bacteriophage and bacteria are being studied. Our results indicate that, as suggested by the disruptive local physical events accompanying electron capture, ^{125}I decay in DNA is much more highly lethal to phage and bacteria than ^{32}P decay under similar storage conditions. When ^{125}I is incorporated specifically into the DNA of *E. coli* 15 THU and the cells are stored at -196° C during decay, approximately 20% of all decays are lethal, compared with 2% for ^{32}P decay. When ^{125}I is incorporated into the DNA of bacteriophage T1 under the same storage condi-

tions, at least 35% of all decays are lethal compared with 5% for ^{32}P decay. The lethal efficiency of ^{125}I decay in bacteriophage may actually be even higher than 35%, because this figure assumes nondiscriminatory uptake of iododeoxyuridine and thymidine, and there is a possibility that the phage actually discriminate in favor of thymidine. Experiments are under way to measure directly the ^{125}I content of radioactive phage particles. Our results also indicate that the lethal effects of ^{125}I decay in phage are much less dependent upon storage temperature than are the lethal effects of ^{32}P decay. If the storage temperature is increased from -196°C to $+4^\circ\text{C}$, the lethal efficiency of an ^{125}I decay changes from about 35% to 45% but that of a ^{32}P decay more than doubles, from 5% to 12%.

CONCLUSION

Our preliminary comparison of the lethal effects of ^{32}P and ^{33}P decay in bacteria and bacteriophage shows no difference between the two isotopes. This strongly suggests that nuclear recoil does not play a major role in causing lethality from ^{32}P decay as proposed by Stent and Fuerst,⁽¹⁾ because ^{33}P decay involves much lower recoil energies, incapable of breaking chemical bonds. The lethal effects must be due, instead, largely to local decay effects which the two isotopes have in common. Likely possibilities are atomic excitation or local production of free radicals.

THE ORGANIZATION AND FUNCTION OF THE BACTERIAL CHROMOSOME. REGULATION OF CHROMOSOME REPLICATION IN *BACILLUS SUBTILIS*. THE EFFECT OF AMINO ACID STARVATION IN STRAIN 168

James C. Copeland

PURPOSE AND METHODS

Regulation of chromosome replication has been studied most extensively in *Escherichia coli*.⁽¹⁻⁵⁾ Starvation of cells for an essential amino acid results in chromosomes aligned at their terminus.^(2,4-6) This has been interpreted to mean that each successive round of chromosome replication is triggered (switched on) by an initiation event which requires a protein to be effected. Once initiated, chromosome replication continues until that round is completed at the terminus of the chromosome.^(7,8) Thus the essential control point for chromosome replication is at the initiation step of replication.⁽⁵⁾ Recently, conflicting reports concerning the effect of amino acid starvation on chromosome replication in *E. coli* have appeared.⁽⁹⁾

The study of the lethal effects of ^{125}I decay in the DNA of bacteria and bacteriophage shows that ^{125}I decays are approximately 10 times more efficient in killing microorganisms than are ^{32}P decays. These results are compatible with the destructive effects observed from decay by electron capture in small organic molecules compared with very mild molecular effects observed from beta decay.⁽⁶⁾ The relative temperature independence of the lethal efficiency of ^{125}I decay also fits in with the concept of direct physical disruption of the DNA molecule in which the decay occurs.

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It is the purpose of this report to show that regulation of chromosome replication in *B. subtilis*, Strain 168,⁽¹⁰⁾ in response to starvation for a required amino acid, differs from that in *E. coli*. This difference allows examination of another regulatory circuit that affects DNA synthesis, and may involve the ratio of DNA to cell mass.

Because *B. subtilis* is transformable, it is possible to study chromosome replication and organization at the molecular level in a direct way. The methods involve transferring cells in balanced growth in a defined, minimal medium to the same medium lacking an essential amino acid. Samples are taken and viability, the amount of DNA and RNA, as determined by the diphenylamine and orcinol tests respectively, is measured. To specifically obtain that DNA made

after a given treatment, such as that made following amino acid starvation, cells are transferred to a medium made up in D_2O . The isolated DNA is fractionated in a CS_2Cl_2 gradient and the fractions are subsequently analyzed, by transformation, for specific genetic marker activity. In this way, the marker activity associated with the newly replicated DNA can be measured and the configuration of the chromosome after the treatment inferred.

PROGRESS REPORT

Cells of *B. subtilis*, Strain BC200, which has requirements for thymine, leucine, and methionine, have a mass-doubling time of 60 to 70 min when grown in a minimal medium at 34°C. Figure 124 shows that cells in balanced growth are not disturbed appreciably by filtering and washing. The cultures deprived of leucine increases slightly in optical density and then stop growing. Leucine starved cells remain viable. The kinetics of macromolecular synthesis during amino acid starvation are shown in Figure 125. In this analysis, the change in optical density is equated with change in total protein. The kinetics of DNA synthesis are unlike those expected if only rounds in progress are allowed to complete, and subsequent rounds are blocked. During amino acid starvation, DNA synthesis continues until 35 to 40% more is

FIG. 124 Effect of starvation of BC200 for leucine. Cells in balanced growth filtered and washed with minimal medium minus leucine. The amino acid was added back to the control, and it was kept below 1×10^8 cells/ml by periodic dilution. ○, Control and ●, cells starved for leucine.

FIG. 125. Total DNA, RNA, and protein measurements on BC200 during starvation for leucine. Arrows indicate when cells were filtered and washed. ○, Cells without leucine; ●, cells with leucine added back. Broken line in DNA curve is the average base line at the time of filtering. The average amount of DNA at time zero is $0.34 \mu\text{g/ml}$, RNA is $1.98 \mu\text{g/ml}$, and optical density for protein is 0.014.

made. There follows a temporary plateau, after which there is a secondary rise in total DNA by an average increase of 70% to a second and permanent plateau.

Readdition of leucine after 150 min of starvation leads to an immediate increase in cell growth paralleled by a similar increase in viable cell number. The kinetics for DNA, RNA, and protein synthesis upon restart are shown in Figure 126. No DNA is made for the first 40 to 50 min, whereas RNA and protein synthesis begin immediately upon restart. DNA synthesis is discontinuous; a plateau occurs when 20 to 25% more DNA is made. This amount, added to the amount of DNA made during amino acid starvation, is equivalent to a doubling, relative to that quantity present at the onset of amino acid starvation. DNA synthesis begins again at about that time when RNA and protein have doubled after restart.

The DNA made after restart in D_2O was separated in a CS_2Cl_2 density gradient. The fractionated gradient was analyzed genetically by transformation to identify the replicating parts of the chromosome. In this analysis, twelve different markers were assayed (Figure 127) in each of four different gradients. The gradients were derived from samples taken when DNA increased 10, 13, 22, and 55 percent after restart. In Figure 128 is represented the percent replication per gradient for each genetic marker vs. the

FIG. 126.—Restart kinetics for DNA, RNA, and protein. BC200 starved for leucine for 150 min, as indicated in Figure 125, at which time leucine was added back to a final concentration of 50 $\mu\text{g}/\text{ml}$. The average amount of DNA at time zero is 0.63 $\mu\text{g}/\text{ml}$, RNA is 2.20 $\mu\text{g}/\text{ml}$, and optical density for protein is 0.053.

FIG. 127.—Representation of genetic map for *B. subtilis* adapted from Dubnau et al.⁽¹⁴⁾

amount of DNA synthesized when the sample was taken. It is readily apparent that upon restart all of the markers assayed were actively replicating. The amount of replicating activity for each marker differs quantitatively in a particular way which will be discussed later. Another characteristic property of all markers is that their replicating activity falls toward zero after about 20% more DNA has been made. This is consistent with the kinetic results obtained on restart of DNA synthesis, i.e., there is a plateau in DNA synthesis when 20 to 25% DNA is made.

Another observation made from these data, but better illustrated in Figure 129, is that the markers appear to be replicating but not in the sequence ex-

FIG. 128.—Replication activity expressed as percent of total first round after replication per genetic marker per gradient after addition of leucine to amino acid starved BC200. This is computed from gradients assayed by transformation as illustrated in Figure 129. The first round after restart is defined as transfer of transforming activity to the hybrid region. When the sum of activity under the curve equals 100%, it would indicate that all copies of a given genetic marker present at restart have replicated once. The relationship is expressed simply as:

$$\% \text{ replicating activity} = \frac{LH/2}{LL + (LH - HH)/2 + HH/2} \times 100,$$

where LL is equivalent to the transforming activity in the light fraction, LH with that in the hybrid fraction, and HH with that in the heavy fraction, each expressed as percent of total transforming activity in the gradient.

FIG. 129.—Transformation assay of gradient taken when 55% DNA has been synthesized after restart. Cells of BC200 were previously starved of leucine for 150 min. LL indicates peak of light fraction, HL that for the hybrid fraction, and HH for the heavy fraction.

ected from their map position. For example, the *his* A marker has been replicated more than the *pur* B marker. The known map order is *pur* A, *pur* B, then *his* A (see Figure 127), but the apparent order of replication after amino acid starvation is *pur* A, *his* A, then *pur* B. Also, the map order is *pur* A, *arg* C, then *lys*, but the apparent order of replication after amino acid starvation is *pur* A, *lys*, then *arg* C. Other misordering can be seen in Figure 128.

To demonstrate that replication is not proceeding in a random order after restart, replicating activity for each marker after 22% DNA has been synthesized is compared to activity when between 22 and 55% DNA has been synthesized, as shown in Figure 130. Initially, replicating activity is greatest on the last half of the chromosome. The noticeable exception is at the *pur* A marker, near the known origin. This activity represents newly initiated chromosomes. Later, replicating activity is lowest on the last half of the chromosome and highest on the first half. These data indicate that replication after amino acid starvation is ordered, and reflects the state the chromosome comes to as a result of the amino acid starvation.

CONCLUSIONS

Regulation of chromosome replication in *B. subtilis*, Strain 168 in response to amino acid starvation is different from that in *Escherichia coli*. In *B. subtilis* 168, chromosomes appear to complete their replication in the absence of the amino acid if one assumes that the near 40% more DNA made to the first plateau is the amount needed to complete rounds in progress.⁽¹⁾ Some of the completed chromosomes, however, then initiate a new round of replication that appears to stop with a broad distribution of resting sites located mainly over the last half of the chromosome. This accounts for the additional DNA made beyond that needed to just complete rounds in progress. This arrangement would best account for the pattern of gene replication found upon restart.

The observed mis-order of markers, when arranged according to their replicating activity compared to their map order, might be accounted for in at least two ways. It could indicate that there are preferred stopping sites for replication forks not able to proceed to the normal terminus. The other possibility is that new rounds of replication can be initiated at sites other than the normal origin of the chromosome, recently demonstrated in *E. coli*.⁽¹⁸⁾

One striking feature of these data stems from consideration of the ratios of DNA to RNA and protein before, during, and after amino acid starvation.

FIG. 130. Bar graph illustrating the distribution of replication activity for genetic markers according to their order on the genetic map of *B. subtilis*. The lower section represents the distribution after 22% DNA synthesis, which is equivalent to the first plateau upon restart. The upper section represents the distribution for DNA synthesis afterwards to 55%, i.e., replication activity at 55% DNA minus replication activity at 22% DNA synthesized.

During amino acid starvation, the amount of RNA drops slightly and protein changes little, or not at all. But DNA is made until there is about 70% more, creating an imbalance in the ratios of DNA to RNA and protein, relative to the amount present in cells during balanced growth. Upon restart, the complementary situation results in RNA and protein being made in the absence of DNA synthesis. As more RNA and protein are made, and normal ratios are approached, DNA synthesis is switched on again. Genetic analysis indicates that those rounds that were in progress continue at the same time as new rounds are begun. Then DNA synthesis and replicating activity shut off when exactly a doubling in amount of DNA is reached, relative to that present at the time amino acid starvation began. It does not begin again until the normal ratios are obtained by continued RNA and protein synthesis. DNA synthesis, thereafter, appears to be continuous and, together with RNA and protein synthesis, the cells appear to be back in balanced growth. These observations are taken as support for a concept first suggested by Maaløe^(5, 13) and more recently extended by Donachie,^(11, 12) that DNA synthesis is regulated by its relationship to RNA, protein, cell mass, or other factors that contain some or all of the former. (For convenience this relationship will be referred to as the DNA/mass

ratio.) One particular proposal relates the initiation event to a definite DNA/mass ratio or a multiple of that mass.⁽¹¹⁾

It is evident from these results that the regulatory circuit involving the DNA/mass ratio can operate independently of the initiation event which controls the onset of replication. New rounds of replication, started by addition of the amino acid, are stopped, as shown by the plateau in amount of DNA made and the concurrent sharp decrease in replicating activity, particularly for the *pur* A marker. DNA synthesis does not begin again until the normal DNA/mass ratio is attained.

It should be emphasized that chromosome replication, once begun, need not go to completion in an uninterrupted sequence. This is clearly shown during amino acid starvation, when newly initiated rounds eventually stop before reaching the terminus. Also, upon restart, newly initiated rounds stop replicating activity until a balanced ratio of DNA/mass is reached. Furthermore, initiation events occur when cells are in an unbalanced condition. This happens during amino acid starvation after rounds in progress come to completion, during which time total RNA and protein has changed only slightly. Again, new initiations occur upon restart before the balanced ratio of DNA to mass is reached. It may be this uncoupling of the initiation event from the control circuit, involving the ratio of DNA to mass, that makes *B. subtilis* 168 different from *E. coli* in its response to amino acid starvation.

The results of this study suggest there are *at least* two regulatory circuits that affect chromosome replication, and affect it differently. Initiation of replication is an all or none event; it acts only once during a round of replication. Once initiated, a given round of replication tends toward completion to its terminus. The second circuit is operative during chromosome replication and modulates replication activity by *turning it on* and *off* relative to other cellular factors that involve cell mass or correlate with it. This

second circuit could serve to entrain chromosome replication to other cellular events, such as cell growth and division.

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THE ORGANIZATION AND FUNCTION OF THE BACTERIAL CHROMOSOME. THE CHROMOSOME IN COMPETENT CELLS OF *BACILLUS SUBTILIS*, STRAIN 168

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PURPOSE AND METHODS

Cells that can take up exogenous DNA are said to be *competent*. Under appropriate conditions, this uptake results in the formation of genetic recombinants. In a competent population of *Bacillus subtilis*,

cells that are competent comprise a minority component. Recently, it was discovered that competent cells are less dense than noncompetent cells,⁽¹⁾ and could be selectively enriched on a step gradient of Reno-grafin.⁽²⁾ This process makes possible a direct study

of competent cells separated from the more numerous noncompetent cells. Studies with mixed populations of competent and noncompetent cells have suggested that the competent population is physiologically re-
sistive. In this study we want to determine the state of the chromosome in this population, i.e., is it in a complete form, as has been postulated for the chromosomes in spores?

Erickson and Braun⁽¹³⁾ previously demonstrated a correlation between the efficiency of transformation of a given marker and chromosome replication. They found that the maximum efficiency of transformation occurred at different times for different markers in a competent population treated so as to have aligned or synchronized chromosome replication. Furthermore, the order in which genetic markers were maximally transformed was the same as the sequence in which these markers are known to replicate. It is the purpose of this study to determine the configuration of the chromosome in the competent population and to further examine the relationship between transformation efficiency and replication.

PROGRESS REPORT

The concentration of Renografin is critical to the relative purity of the lighter competent fraction of cells. The data illustrated in Figure 131 show that, as the density of the separating layer of Renografin is increased, more cells remain at the top of the gradient; at the same time, DNA binding per cell decreases sharply. Thus, in the experiments to be described, Renografin solutions diluted to a refractive index of 3.3680 ± 0.0005 were used as the separating layer.

The commercial preparations of Renografin used in these studies contained two preservatives (methyl paraben and propyl paraben) as well as inhibitory concentrations of disodium EDTA dihydrate. To obtain a physiologically active cell population after separation on Renografin required a vigorous, yet non-lethal, washing procedure. This involved collecting the cells on a Millipore filter (pore diameter 0.45μ) and washing them in growth medium containing $0.002 M Mg^{++}$, followed by a wash in $0.02 M Mg^{++}$ in D_2O and a final wash in the D_2O medium containing $0.002 M Mg^{++}$ and 10% glycerol. The cells were then fast-frozen and stored at $-70^\circ C$. Individual preparations of competent cells were pooled to provide a sufficient concentration of cells for further examination.

To analyze relative rates of DNA synthesis in competent and noncompetent cells during the incubation period required to produce a competent population, the cells were exposed to 3H -labeled thymidine at different times before separation on Renografin. The

FIG. 131. Relationship between the refractive index of Renografin and the efficiency of separation of competent *B. subtilis* cells. 3H DNA was added to a competent culture for 30 min, the reaction stopped by deoxyribonuclease, and cells separated on a step gradient of Renografin. $\circ$ $\circ$, DNA bound to cells; $\bullet$ $\bullet$, percent viable cells; and Δ Δ , transformants in top fraction.

FIG. 132. The incorporation of 3H thymidine into top (competent) and bottom (noncompetent) cell fractions. Solid bars represent the competent cell fraction. Open bars represent the noncompetent cell fraction.

cells were then fractionated, and the distribution of label in the two cell fractions (competent and noncompetent) was determined. The results, illustrated in Figure 132, show that DNA synthesis is decreasing in the competent cells but increasing in the noncompetent cells during this incubation period. This implies that competent and noncompetent cells are physiologically different, in agreement with published reports.⁽¹⁴⁾

The kinetics of DNA, RNA, and protein synthesis in the fractionated competent cells grown in an enriched D_2O medium are illustrated in Figure 133. As

FIG. 133.—Kinetics of macromolecular synthesis in competent cells grown in enriched D_2O medium at $34^\circ C$. ●, DNA; ○, RNA; and ◐, protein as total mass.

FIG. 134. Replication map of the *B. subtilis* chromosome.⁽¹⁰⁾

has been found by others,⁽⁴⁾ DNA and RNA synthesis is low, relative to the increase in mass of the culture. DNA does not appear to increase continuously during this growth period.

The newly replicated DNA made in the D_2O medium was isolated and fractionated on the $CsCl$ density gradient. The gradients were then assayed for transforming activity.⁽⁵⁾ Genetic activity associated with replicated DNA is found in the fractions that contain hybrid density DNA and that associated with unreplicated DNA is found in the fractions that contain the light-density DNA (Figures 134-136). According to this analysis, the terminal half of the chromosome is replicating most actively. Thus, chromosome replication is aligned in competent cells, but not at a defined replication origin (near the *purA16* locus). The majority of chromosomes appear to have replication points distributed between *ura* and *lys* when the competent cells were separated. It is interesting to note that this pattern of alignment is like

that obtained when cells of Strain 168 are starved for an essential amino acid.⁽⁶⁾ What significance this observation has concerning competence and/or regulation of chromosome replication is highly speculative at this time.

The pattern of replication observed could be explained by competent cells having half-completed chromosomes which continue replication when the cells are placed in a fresh growth medium, or by new initiations occurring on the terminal half of the chromosome. The gradient method does not allow a choice between these alternatives, but the marker frequency

FIG. 135.—Distribution of transforming activity for different markers in fractions of $CsCl$ gradient. DNA obtained from competent cell fraction isolated from H_2O medium and transferred to D_2O medium. Total increase in DNA synthesized at sampling time was 22%. HL indicates hybrid density and LL indicates light density fractions.

FIG. 136.—Distribution of transforming activity for different markers in fractions of $CsCl$ gradient. DNA obtained from competent cell fraction isolated from H_2O medium and transferred to D_2O medium. Total increase in DNA synthesized at sampling time was 51%. HH indicates heavy density, HL indicates hybrid density, and LL indicates light density fractions.

TABLE 70. MARKER FREQUENCY ANALYSIS

Strain	Markers	Observed marker frequency ratio		Normalized value ^(a)
		Spore DNA	Competent cell DNA	
BC100	<i>purA16 metB</i>	1.16	2.36	2.03
BC35	<i>purA16 metB</i>	0.311	0.488	1.57
BC36	<i>thr metB</i>	0.940	1.41	1.53
BC34	<i>ura metB</i>	0.169	0.249	1.47
BC50	<i>nia metB</i>	0.606	0.852	1.41
I-64	<i>trp metB</i>	0.191	0.197	1.03

^(a) [(1.00) (Marker frequency ratio obtained with spore DNA)] $\times$ [Marker frequency ratio obtained with competent cell DNA] = [Normalized value].

analysis does.⁽⁷⁾ In this analysis the frequency of genetic markers in a DNA preparation is determined relative to a reference marker. To correct for differences in efficiency of transformation between markers these relative values must be compared to a sample with equal frequencies for all markers, i.e., spore DNA.⁽⁸⁾ To apply this technique successfully, all genetic markers must be isolated without preference. Thus, a DNA preparation was used where no part of the sample was discarded. Removal of low molecular weight breakdown products was accomplished by dialysis. As an additional precaution, the DNA con-

centration from the cell sample and that of the spore DNA preparation were adjusted to give approximately equal numbers of transformants for a given genetic marker. It was observed that unequal DNA concentrations produced anomalous marker frequency ratios, presumably because the slopes of the plots of DNA concentration vs. frequency of transformation are not equal for the different markers. In fact, this relationship may not be linear for some genetic markers.

Results of the marker frequency analysis, shown in Table 70 agree with the gradient analysis and confirm that the competent *B. subtilis* cell possesses a partially completed chromosome which has initiated replication from the defined origin. This is indicated by the observation that the normalized *purA16 metB* ratio is highest with no normalized marker ratio exceeding that of a marker preceding it on the replication map (Figure 134). The very abrupt drop in the normalized marker ratio, between *purA16 metB* and *purB6 metB*, suggests the initiation of a new round of replication, perhaps during the washing and separation procedures. In addition, the very gradual decline in the normalized marker frequency, between *purB6 metB* and *nia metB*, and the second abrupt drop, between *nia metB* and *trp metB*, indicate that the majority of chromosomes have been replicated at least as far as *ura* and *nia*.

As demonstrated in the gradient analysis, the ter-

FIG. 137. Model for chromosome replication in competent cells of *B. subtilis* 168. LL designates DNA synthesized in H_2O medium—both strands of light density. HL designates DNA synthesized once in D_2O medium—parental strand of light density and daughter strand of heavy density. HH designates

DNA synthesized twice in D_2O medium—both strands of heavy density. —, DNA made in H_2O medium and - - -, DNA made in D_2O medium. O, origin of chromosome replication; T, terminus of chromosome replication.

minimal portion of the chromosome replicates first and then the proximal part, to the extent that all markers are equally represented in the once-replicated DNA (see Figure 136) when DNA synthesis has increased by approximately 50%. It is important to note that the genetic markers replicated first are now beginning to replicate a second time (their activity is apparent in the heavy fractions). This is true for all markers but the *purA16* marker, in accord with a marker frequency analysis, which indicates that some chromosomes have replicated during the separation procedure and before transfer to the D₂O medium. All of these observations, taken together, suggest that the competent cell contains one half-replicated and one fully replicated chromosome. During the separation procedure, one arm of the partially replicated chromosome initiates and replicates at least to the *purA16* site. Upon transfer to the D₂O medium, the half-replicated chromosome is completed, followed closely by the completion of the already initiated sister chromosome, before subsequent initiations and replication from the origin occur (which accounts for the *purA16* marker activity in the hybrid density fractions). This is represented diagrammatically in Figure 137 and forms part of a proposed model to explain competence in *B. subtilis*.⁽⁹⁾

The joint transformation of two unlinked genetic markers has been taken as a measure of the fraction of the total population that is competent, since it is assumed to be a function of two, random independent events.⁽¹⁰⁾ However, if transformation is influenced by replication, as shown in an earlier study,⁽³⁾ this assumption would be invalid. This rela-

FIG. 138.—Changing efficiencies of transformation in competent cell fraction during incubation with donor DNA. Relative values obtained by subtracting relative increase of all classes of transformants from relative increase for a given marker. Δ , *met*⁺ transformants; $\circ$, *thr*⁺ transformants; and $\bullet$, *leu*⁺ transformants.

TABLE 71. MAPPING ACCORDING TO SELECTION OF DOUBLE TRANSFORMANTS

Genetic markers	Frequency of single transformations	Frequency of double transformations	$\frac{f(a)f(b)^{(a)}}{f(ab)}$	Relative ^(b) map distance
(<i>tms-12</i>)	0.00161			
(<i>leu</i>)	0.00232			
(<i>metB</i>)	0.00333			
(<i>tms-12</i>) (<i>leu</i>)		0.0000942	0.0391	9.2
(<i>tms-12</i>) (<i>metB</i>)		0.0000961	0.0550	13.0
(<i>leu</i>) (<i>metB</i>)		0.0000900	0.0843	20.0
(<i>argC</i>)	0.00184			
(<i>leu</i>)	0.000979			
(<i>metB</i>)	0.00140			
(<i>argC</i>) (<i>leu</i>)		0.000115	0.0156	43.0
(<i>argC</i>) (<i>metB</i>)		0.000171	0.0151	41.6
(<i>leu</i>) (<i>metB</i>)		0.000189	0.00725	20.0
(<i>purA16</i>)	0.0536			
(<i>leu</i>)	0.0215			
(<i>metB</i>)	0.0273			
(<i>purA16</i>) (<i>leu</i>)		0.00149	0.772	31.4
(<i>purA16</i>) (<i>metB</i>)		0.00413	0.354	14.4
(<i>leu</i>) (<i>metB</i>)		0.00119	0.493	20.0
(<i>ile</i>)	0.00522			
(<i>leu</i>)	0.00654			
(<i>metB</i>)	0.00532			
(<i>ile</i>) (<i>leu</i>)		0.000254	0.134	24.6
(<i>ile</i>) (<i>metB</i>)		0.00135	0.0206	3.8
(<i>leu</i>) (<i>metB</i>)		0.000320	0.109	20.0
(<i>purB6</i>)	0.00277			
(<i>leu</i>)	0.00173			
(<i>metB</i>)	0.00241			
(<i>purB6</i>) (<i>leu</i>)		0.0000806	0.0594	37.0
(<i>purB6</i>) (<i>metB</i>)		0.000255	0.0262	16.3
(<i>leu</i>) (<i>metB</i>)		0.000130	0.0321	20.0
(<i>thr</i>)	0.0517			
(<i>leu</i>)	0.0239			
(<i>metB</i>)	0.0167			
(<i>thr</i>) (<i>leu</i>)		0.00257	0.482	39.2
(<i>thr</i>) (<i>metB</i>)		0.00278	0.310	25.2
(<i>leu</i>) (<i>metB</i>)		0.00162	0.246	20.0
(<i>nia</i>)	0.0232			
(<i>leu</i>)	0.0137			
(<i>metB</i>)	0.0227			
(<i>nia</i>) (<i>leu</i>)		0.00170	0.0187	9.6
(<i>nia</i>) (<i>metB</i>)		0.00178	0.0296	15.2
(<i>leu</i>) (<i>metB</i>)		0.000800	0.0389	20.0
(<i>ura</i>)	0.00163			
(<i>leu</i>)	0.00215			
(<i>metB</i>)	0.00273			
(<i>ura</i>) (<i>leu</i>)		0.0000791	0.0442	17.9
(<i>ura</i>) (<i>metB</i>)		0.0000879	0.0506	21.0
(<i>leu</i>) (<i>metB</i>)		0.000119	0.0493	20.0

^(a) $f(a)$ and $f(b)$ are the frequencies obtained for single marker transformations for markers a and b selected independently. $f(ab)$ is the frequency obtained for double marker transformation for markers a and b selected simultaneously.

^(b) Total genetic map taken as 100 units; the distance between *leu* and *metB* taken as 20 units (from Dubnau et al.⁽¹¹⁾). All other genetic markers assigned map unit values relative to *leu* or *metB*.

relationship was examined again, but with the isolated competent fraction of cells in which the subsequent order of chromosome replication has been determined, as already described. It is to be emphasized that these cells were in no way manipulated purposefully to align chromosomes, other than to make them competent. The results are represented in Figure 138 and clearly show that efficiency of transformation for a given marker changes with time and that the sequence in which they reach their maximal efficiency for transformation is the same as the order they replicate in the competent cell. Thus, the probability of a successful double-transforming event should increase as a function of the proximity of the two loci to an approaching replication fork. This relationship would then reflect the order in which the genetic markers replicate and could be used to generate a genetic map. Table 71 presents the data for all possible classes of double transformants among the markers selected. It is obvious that the value of double transformants varies significantly in relation to the markers chosen. If this relationship is used to generate a genetic map relative to two arbitrarily chosen standard markers, the remaining genetic loci are located in an order that agrees with the order found in already published genetic maps for *B. subtilis* (Figure 139).⁽¹¹⁾ Genetic maps designed as a function of double transformants do not reflect accurate linkage relationships, but do

present the order in which the markers replicate. These results clearly show that the efficiency of transformation is influenced by the replication process and confirm and extend the earlier observations.

CONCLUSIONS

Competent cells of *B. subtilis* contain one chromosome that is half-replicated and another that is fully replicated. The replication pattern produced by placing competent cells in a medium for growth is strikingly similar to that obtained when amino acid-starved cells of Strain 168 are released from starvation. An association between transformation and replication has been confirmed.

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FIG. 139. Genetic maps of *B. subtilis* chromosome. A, transformation map based on estimated distances from *metB*; B, linkage map of Dubnau et al.⁽¹¹⁾ arranged in the order determined by the transformation mapping function; C, transformation map based on estimated distances from *leu*; D, map distance based on calculations of O'Sullivan and Sueoka⁽¹²⁾; E, all maps based on assumption that distance from *leu* to *metB* is equal to 20 map units.

METABOLIC AND THERAPEUTIC STUDIES OF PLUTONIUM. V.

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PURPOSE AND METHODS

The broad objective of this program is the development of new approaches to the therapy of poisoning by radioactive and nonradioactive metals.⁽¹⁾ Plutonium-239 has received increasing emphasis in this work during the last several years,⁽²⁾ because its growing use in public power reactors and its high radiotoxicity in bone make it an important radiological health hazard. The varying tendency of compounds of plutonium (as well as other polyvalent heavy metals) to hydrolyze and polymerize, both in solution and *in vivo*, results in variable particulate characteristics which influence the deposition, retention, effectiveness of therapy, and delayed pathological effects of the plutonium. Thus, information obtained with plutonium aids in understanding the behavior of other nuclides of the actinide, lanthanide, and rare earth series in living tissues. In addition, plutonium compounds provide useful information regarding the translocation and deposition of colloids and macromolecules in tissues.

Following the demonstration of the effectiveness of chelating agents such as diethylenetriaminepentaacetic acid (DTPA) for removal of plutonium and related elements from blood, bone, and soft tissues, recent attention has been directed toward the development of additional therapeutic approaches to the removal of the plutonium not readily removed from liver and bone by DTPA.

Related to the plutonium program is a concomitant investigation^(3, 4) concerned with the normal processes of calcium uptake and depletion and the biochemical transformations in the calcifying matrix of preosseous cartilage. This study is designed to provide a better understanding of the uptake of metal cations, including such diverse radionuclides as plutonium and strontium, in cartilage and bone.

This report describes recent studies of the effects of the physical form of administered plutonium on distribution and retention in tissues and on long-term effects, the estimation of plutonium deposited in bone marrow and in cellular and subcellular ele-

ments in the liver, and adjunct therapy with glucan to remove hepatic plutonium not accessible to the action of DTPA.

PROGRESS REPORT

Effects of Physical Form of Plutonium on Tissue Distribution and Retention and on Long-Term Effects

The study of the influence of the physical-chemical properties of injected plutonium on the long-term effects of deposited plutonium is a continuing project. Previous work has shown that, with equal amounts in the mouse skeleton, polymeric plutonium is less carcinogenic than monomeric plutonium, presumably because the smaller fraction deposited on bone surfaces reduces the amount of radiation delivered to endosteal cells.⁽⁵⁾ To explore these relationships further, an experiment is in progress in which equal initial skeletal burdens were achieved in two series of mice given a single intravenous injection of ²³⁹Pu. The first series received graded polymeric plutonium of narrow particle size range prepared by a differential precipitation technique;⁽²⁾ the second series received monomeric plutonium of <0.01- μ m particulate diameter (Table 72). Although only one-fourth of the mice were dead by 400 days, a summary of the results obtained so far is of interest. The tissue distribution and retention of the two forms of plutonium have been determined in mice designated for the purpose and selected at random for serial sacrifice at 1, 3, 6, 20, 44, 90, 181 and 350 days.

After injection of the *monomeric* form, distribution and retention of the plutonium were generally comparable to those described earlier for an unfiltered monomeric plutonium,⁽⁵⁾ except for a slightly higher initial bone burden. At 6 days, 45% of the injected dose was in the bone. It is estimated from studies with iron-59 (reported below) that less than 1/20 of the total bone burden was in the marrow.

After injection of the *polymeric* plutonium, the high uptake in liver and spleen to be expected with this form⁽²⁾ was observed. At 6 days, these organs

contained 84% and 6% of the amount injected, respectively, while the total skeletal burden was 3%. Almost two-thirds of this bone burden is estimated from the iron-59 studies to be in the marrow. In the group with the highest level, at 6 days, the specific activity of plutonium in the liver and spleen was about 0.14 and 0.12 $\mu\text{Ci/g}$, respectively, but in the marrow it was estimated to be less than 0.01 $\mu\text{Ci/g}$ in spite of the high relative concentration of plutonium in marrow compared to hard bone. Between 6 and 350 days, the spleen burden did not change, although the liver burden decreased by about one-third, and the bone burden, as represented by the femurs, increased about fivefold. This translocation to bone confirms earlier observations.⁽²⁾

In the dose ranges used for this experiment, the amount of either form of plutonium injected did not influence the fraction deposited in any tissue.

The serially-sacrificed mice also were used for histopathologic, autoradiographic and limited hematologic studies.* The remainder of the mice were maintained for duration-of-life studies and sacrificed when death appeared imminent. At 400 days, survival of these long-term mice was either the same as, or higher than, the controls, except in the two groups that received the highest levels of plutonium.

The 52 duration-of-life mice that received the highest level of polymeric plutonium (7.8 $\mu\text{Ci/kg}$) died between 128 and 254 days; the primary cause of death in all mice apparently was anemia. Because death specifically attributed to anemia is most unusual after plutonium administration,⁽⁶⁾ the results to date from this group will be summarized in more detail. Included are observations on 47 mice sacrificed from 1 to 181 days and on the 52 duration-of-life mice.

Evidence of possible hematopoietic damage was first suggested by the white cell count at 90 days, which was about one-third of the control value. Leukopenia was present in all mice at the end of life, as indicated by the almost complete absence of white cells in blood smears and by the extremely thin buffy coat. There was no evidence of septicemia. Hemoglobin and hematocrit values in individual serially-sacrificed mice at 181 days ranged from normal to about one-half normal. All long-term mice were markedly anemic at end of life: in the 17 mice sampled from 128 to 254 days, the mean hemoglobin was 1.9 g/100 ml, compared to 17 g/100 ml in controls, and the mean hematocrit was 5.4%, compared to 46% in controls.

Liver degeneration occurred in all mice of this group after 20 days. At 44 days, clear-cut hydropic degen-

TABLE 72. INJECTED DOSE AND BONE CONTENT OF ^{239}Pu AFTER INTRAVENOUS INJECTION INTO 100-DAY OLD CFI/Adl FEMALE MICE

	Polymeric plutonium					Monomeric plutonium				
$\mu\text{Ci/kg}$ injected	1.1	2.2	3.8	7.8	0.07	0.14	0.27	0.47	0.96	
nCi in bone at 6 days	0.9	1.7	3	6	0.8	1.6	3	5.3	10.8	

erative changes were noted histologically. The degeneration was mainly in the centrilobular regions, but in more severely damaged livers there was diffuse degeneration and coagulation necrosis. At 90 and 181 days, and in all duration-of-life mice examined, these lesions were of the same nature but more severe; the severity was not correlated with time. No regenerative tissue was noted at any time.

Atrophy of the spleen was a consistent change. At 6 days, the spleen weight was slightly reduced, and by 20 days it was one-half of the control weight. The mean spleen weight of the duration-of-life mice was 8.7 mg, compared with a control weight of 100 mg. Atrophy, with some loss of the lymphoid follicles, was seen at 44 days. By 90 and 181 days, there was almost complete aplasia of the lymphoid tissue, but sinusoidal and reticular tissues were retained. A few granulocytes were seen in 2 spleens. In the spleens of duration-of-life mice, lymphoid elements were absent, except in a few small nodules under the capsule in some mice. These nodules were probably the same as those seen in varying numbers in half of these mice at autopsy. Whether these nodules represent residual or regenerative lymphatic tissue is not clear.

Although the lymph nodes and thymus of many of the duration-of-life mice were small, fairly large lymphoid aggregations were present and increased numbers of plasma cells were seen in some mice. Hemorrhages or hyperemia with dilated vessels or sinusoids congested with erythrocytes were often seen in the nodes. Ectopic hematopoiesis, involving erythropoietic and myelopoietic elements, was seen in the nodes of 7 of the 16 mice examined; this was more common in mice that survived 200 days or more. The myelopoiesis is considered compensatory rather than neoplastic because of the presence of both erythropoietic cells and megakaryocytes.

The kidneys were comparable to those of control mice, except for ectopic hematopoiesis in one kidney from a duration-of-life mouse at 196 days.

Other characteristic findings in this group included enlarged, sometimes hemorrhagic hearts and petechial hemorrhages, usually in the skin.

* We gratefully acknowledge the advice and cooperation of Dr. Thomas E. Fritz and Dr. Carl E. Rehfeld in these phases of the work.

The bone marrow was severely damaged after 7.8 $\mu\text{Ci/kg}$ of polymeric plutonium, as shown by blood studies and by the death of all duration-of-life mice with anemia. Bone sections and marrow prints of a terminal mouse at 133 days showed general aplasia of marrow, but there was considerable variability among individual bones.

Marked increase in skeletal density, shown by increased radio-opacity on the roentgenograms, was characteristic of these mice. Histologically, increased numbers and thickness of trabeculae and thickened cortical bone were obvious.

No bone tumors were observed in the mice of this group; most had died before the expected latent period was over.

With lower levels of polymeric plutonium, less hematopoietic damage was seen. From about 300 days, hematopoietic deaths did occur, most commonly in the mice that received 3.8 $\mu\text{Ci/kg}$. However, they also died from other causes, including osteogenic sarcoma.

After monomeric plutonium, osteogenic sarcomas also have been observed. The shortest interval to death with an identified bone tumor was 283 days after plutonium injection. The limited data available indicate that there was probably no significant hematologic effect of the monomeric plutonium through 250 days. No histopathologic lesions were observed during this period.

Localization of Plutonium in Bone and Liver

Measurement of plutonium in marrow and intact bone.—The fraction of plutonium in the marrow of a

FIG. 140 Relationship of iron-59 uptake in mouse tibial shaft marrow to total skeletal uptake at 5 hr after intravenous injection

standard 9.0-mm segment of the mouse tibia has been used as one measure of tissue distribution after the injection of different forms of plutonium.⁽⁷⁾ A marrow index has been proposed, based on the estimation that the amount of plutonium in the marrow of this segment is $\frac{2}{3}$ of the total tibial marrow.⁽²⁾ Measurements of the plutonium in the total marrow and in individual bones are also needed to assess more exactly the roles of bone surface and bone marrow plutonium in the induction of bone tumors. Pending the development of a satisfactory chemical separation of marrow and bone (see below), a method based on the uptake of ^{59}Fe by erythropoietic tissue is being used to obtain this information indirectly.

After intravenous injection of ^{59}Fe in 1% citrate, mice were sacrificed at 5 hr and perfused with saline to remove the blood. Radio-iron uptake was determined in all skeletal parts, including the separated marrow and bone of 9-mm tibial shaft segments. Although the total skeletal uptake of ^{59}Fe (assumed to be equivalent to the marrow uptake) varied widely among individual mice (Figure 140), there was a reasonably constant relationship between the uptake of ^{59}Fe in any one bone and that in the total skeleton. The sample of marrow in the tibia was as reliable as larger samples for calculation of total skeletal iron. Based on the data to date, therefore, an average factor of 43 is used in the mouse for conversion of the marrow in two tibial segments to total-body marrow. The work with radio-iron has also shown that the tibial segment contains about $\frac{2}{3}$ of the total tibial marrow, rather than $\frac{2}{5}$, as estimated earlier.⁽²⁾ To assess the value of radio-iron in computing the distribution of plutonium, further work will include double-tracer experiments with ^{59}Fe and ^{239}Pu .

Because extrapolation to man is a long-range objective of this program, it is important to compare the metabolism of different forms of plutonium in larger species (rabbit and dog) with that in the mouse and rat. In the rabbit, physical separation of marrow from bone for plutonium analysis is simplified in the long bones, particularly in the tibiae, which have a minimum of spongy bone. The more extensive trabeculation in the long bones of the dog, and in the spines of both species, however, prevents complete physical removal of marrow. A continuing search for an effective chemical procedure for marrow removal is therefore in progress. "NCS" (Nuclear Chicago Co.), a quaternary amine dissolved in toluene, is a promising compound for removal of the organic material from halved bones of both rabbit and dog. To determine conditions that will insure minimum removal of plutonium bound to bone surfaces, a com-

parison is being made of chemical and physical removal techniques, using plutonium-injected rabbits.

The liquid scintillation method for plutonium analysis is complicated in the case of large amounts of tissue (e.g., dog femur or liver) by excess amounts of interfering inorganic residues. To avoid this problem, a liquid-liquid extraction technique has been developed, in cooperation with Mr. W. D. Fairman, that uses the organic solvent TIOA (triisooctyl amine) and permits recovery of 90% to 100% of the plutonium added to large masses of synthetic bone ash.*

Microdistribution of Plutonium in Liver.—Autoradiographic studies^(8,9) aimed at the quantitative estimation of plutonium deposits associated with tissue structure at the cellular and subcellular level are continuing. To establish the validity of conversion of alpha track counts in a histological section to specific tissue activity, certain geometrical assumptions are being tested. If these assumptions are correct, autoradiograms prepared from equivalent sections of the same tissue should give the same value for the amount of deposited plutonium, whether determined at different exposure times or whether tracks are counted at optical microscopic or electron microscopic levels. These values should agree with the value for tissue plutonium obtained by gross radiochemical analysis. In preliminary comparisons of autoradiograms of liver, the variance from the gross radiochemical value after 2 or 6 days' exposure was one- to twofold at the optical microscopic level, and two- to fourfold at the electron microscopic level after 3 or 6 months' exposure.

The retention of polymeric plutonium in rat liver is known to involve uptake by at least one intracellular organelle, the lysosome.⁽¹⁰⁾ Further efforts are being made to learn more about the deposition sites and transport of radioactive macromolecules. In particular, the effects of colloidal and particulate properties upon their partition among cellular and extracellular structures and among different cell types in the liver are being studied. A comparison of the retention of monomeric and polymeric plutonium in mouse liver homogenates† indicates that the fractional distribution of injected monomeric plutonium is nearly identical to that of polymeric plutonium. Thus, lysosomal association is indicated for both forms of plutonium. However, about 46% of the total liver content of the *monomeric* plutonium was accounted for in all the intracellular components measured, whereas only

about 7% of the *polymeric* plutonium was similarly identified; most of the polymeric plutonium was associated with liver components denser than mitochondria.

Attempts have been made to isolate intact parenchymal and littoral cell types from rat and mouse liver and to further isolate cell membranes from each of these cell types. These have not been entirely successful because of inadequate techniques. However, the isolation of small numbers of cells of each type and of parenchymal cell membranes has been achieved.‡ Although gross radiochemical analysis of associated plutonium is not feasible, it is hoped that autoradiography of isolated components will yield useful information for application to the problems of transport mechanisms and therapeutic removal.

Therapeutic Studies with Glucan

In accordance with the long-range objective of this program, which is the development of more effective therapeutic procedures, attempts are being made to remove deposited plutonium from tissue sites not accessible to chelating agents such as DTPA. Recent work has demonstrated the effectiveness of glucan, a purified polysaccharide obtained from brewer's yeast (*Saccharomyces cerevisiae*), in accelerating the removal of polymeric plutonium deposited in mouse liver.^(12,11) Both the chemical and physical properties of glucan, including particle size, are now considered to be important parameters in its therapeutic effect. It is, therefore, important to investigate the relation between these properties and the effectiveness of well-characterized glucans obtained from yeast and other sources.

Several batches of brewer's yeast glucan were prepared by a method employing alkaline hydrolysis followed by ethanol-ether extraction.§ Microscopic examination indicates that the product obtained consists nearly exclusively of smooth ovoid particles of approximately 3- μ m diameter. In addition, we have recently received samples of glucans isolated from another organism, the fungus *Sclerotinea*, from Prof. Y. Satomura of Osaka City University, Japan. The toxicity and effectiveness of these substances will be tested in mice injected with plutonium.

Other Related Activities

The problems of distribution, retention and removal from living tissues presented by plutonium are prototypic of a large group of radionuclides which include the actinides, lanthanides, and rare earths. The highly

‡ We thank Elizabeth Cerny for valuable assistance in this phase of the work.

§ This work was carried out by Raymond K. Hertz.

* This work was performed by Raymond K. Hertz, who was a participant in the 1969 ANL Summer Student Training Program.

† This work was performed with the assistance of Johanna Josephson during her tenure as an ACM semester student.

variable tendency of these elements to hydrolyze and polymerize under physiological conditions has resulted in large uncertainties in the estimation of body burdens, determination of critical organs, and dosimetry of individual tissues, both in animals and in human subjects. Accordingly, in 1967 ICRP Committee 2 activated a task group charged with the responsibility of gathering pertinent chemical and biological data on the effect of physicochemical properties on the metabolism of these radionuclides. Two Argonne staff members, Drs. A. Lindenbaum (Chairman) and M. W. Rosenthal are members of this task group. The first draft of a report, entitled "Metabolism of Plutonium and Related Elements, and their Compounds," is now in preparation.

In addition to ICRP activities, one of us (A. L.) is also a member of NCRP Scientific Committee 30, whose task is to prepare a report on the physical and biological properties of radionuclides.

CONCLUSIONS

Protracted hematopoietic deaths occur in mice after injection of $7.8 \mu\text{Ci/kg}$ of *polymeric* plutonium of narrow particle size range which is deposited heavily in the reticuloendothelial organs. The concentration in the marrow was estimated to be less than that in the liver and spleen by an order of magnitude. All mice died of anemia between 4 and 9 months. This anemia was characterized by marrow aplasia and by hematocrit and hemoglobin levels almost $1/10$ of controls. Ectopic myelopoiesis occurred in lymphatic tissues in the later deaths. Leukopenia, hydropic degeneration of the liver, marked splenic atrophy, petechial hemorrhages, and increased bone density were also observed. After lower doses of this polymeric plutonium, hematopoietic deaths occurred at longer intervals, but some mice died from osteogenic sarcomas and other conditions.

Translocation of plutonium from liver to bone after injection of polymeric plutonium has been confirmed.

By use of radio-iron as a tracer for bone marrow in mice, a factor of 43 was obtained for conversion of the marrow of a standard tibial segment to total body marrow. Based on this factor, almost two-thirds of the skeletal burden of this polymeric plutonium is in the marrow, as compared to less than $1/20$ with monomeric plutonium.

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BIOCHEMISTRY AND ION BINDING OF CONNECTIVE TISSUE. IV.

Arthur Lindenbaum and Quenton T. Smith

PURPOSE AND METHODS

In the removal from the body of many toxic radioelements, including the polyvalent, heavy metals, such as plutonium, bone is the critical tissue. A better understanding of normal and abnormal physiological processes in bone, therefore, is of practical as well as fundamental importance. The overall objectives of this project are to investigate the biochemical changes taking place in cartilage during calcification and ossification, particularly with regard to changes in the mucopolysaccharide and mucoprotein components, and to determine the involvement of such substances in the accumulation of normal and abnormal metals such as calcium and plutonium. The objectives of present experiments are 1) to determine whether there are differences between the mucoprotein composition of bovine nasal cartilage (a tissue that does not ossify) and the composition of different regions of calf scapular cartilage associated with the ossification process, and 2) to measure the binding of intact cartilage and of isolated mucoprotein components with calcium and other metal ions.

PROGRESS REPORT

Previous work⁽¹⁾ has been devoted to the isolation of mucoprotein subfractions from bovine nasal septum cartilage and from preosseous cartilage of the resting and hypertrophic zones of calf scapula, using modifications of the extraction method of Schubert and associates.⁽²⁾ These isolated mucoprotein subfractions have now been analyzed with respect to yield, equivalent weight, uronic acid, hexosamine, sulfate, sialic acid, calcium, etc. Except for yield, few differences have been noted.

Possibly significant variations in yield have been found in the PP-L4 and PP-L6 subfractions. Calculated as the fraction of total PP-L extracted, in nasal septum about 10% of the PP-L was PP-L4, as compared with 0.7 to 0.9% in the resting and hypertrophic zones of calf scapular cartilage. Similarly, over twice

as much of the PP-L6 subfraction was found in the hypertrophic zone as in the resting zone of calf scapula. These variations in yield are not associated with appreciable differences in the other analytical values obtained.

In preliminary work concerned with the measurement of affinity constants between cartilage components and metal ions, a comparison has been made between the ion exchange and equilibrium dialysis methods for measuring the binding of calcium to PP-L subfractions of nasal septum and calf scapular cartilage. Problems related to viscosity and concentration appear to be less troublesome with the ion exchange method. Early results, although incomplete, show a low order of binding with calcium ($\log K \sim 2$), with differences of no more than an order of magnitude among all PP-L subfractions. On the basis of present evidence, the amount of calcium bound at each region of ossifying cartilage would appear to depend on the amount of organic ligand available for binding.

This work is being supplemented by parallel studies with plutonium in small animals, using autoradiography to localize the radionuclide at specific sites in cartilage and bone.

CONCLUSIONS

Investigation of the differences in the composition of ossifying and nonossifying cartilage, and the significance of such differences, will be continued, both for a better understanding of metal ion uptake in bone formation and for therapeutic manipulation of radionuclides incorporated in bone.

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RESTORATION OF LETHALLY UV-EXPOSED AND HN2-TREATED AMOEBAE BY TRANSPLANTATION

Edward W. Daniels and Judith M. McNiff

PURPOSE AND METHODS

Recently, a large amoeba found in Colorado was identified as a new strain of *Pelomyxa carolinensis*.⁽¹⁾ We determined its sensitivity to ultraviolet radiation (UV) (254 nm) and nitrogen mustard (HN2) with a view toward using it in studies of radiorestitution and recovery after lethal doses of HN2. It is well known that UV damages DNA in cells, and that nitrogen mustard reacts primarily at the N-7 position of the guanine ring in DNA.⁽²⁾ This exploratory study investigates ways to rescue cells from lethal UV and HN2 injuries. Additional intracellular UV and HN2 damage is also sought, as this knowledge may permit the development of improved therapeutic treatment of radiation injury.

Amoebae were exposed to UV in groups of 10 per 0.5 ml open glass vials, 7 mm in height containing 6

mm glass-distilled water. The amoebae lay on the bottom during the exposures. The UV was emitted by a pen-ray quartz lamp at 254 nm (Ultra Violet Products, Inc., San Gabriel, Calif.; Model No. SCT1; 115 volts, 60 cycle; 0.22 amps). Two dose rates were used (100 ergs mm² sec and 30 ergs mm² sec at target distances of 6.5 cm and 12.5 cm, respectively). All UV-exposed amoebae were kept in similar visible light throughout the experiments to equalize photoreactivation effects.

The nitrogen mustard (HN2) was purchased from Merck, Sharp, and Dohme* as a trituration of Mustargen HCl (methylchloroethamine-HCl) in 10-ml rubber-stoppered vials, each containing 10 mg of the drug with sodium chloride q.s. 100 mg. Amoeba buffer⁽³⁾ (pH 6.9) was used both as a solvent and a culture medium. The time between the addition of the solvent to the drug and the beginning of the treatment was 2.5 ± 0.5 min. The total treatment time was 1 hr, after which the amoebae were washed in buffer and fed by adding food organisms (paramecia and chlomonas).

Whole unirradiated, untreated *P. carolinensis* (Colorado strain), or portions (halves, thirds, quarters) cut from them, were microsurgically transferred (fused) into UV-exposed and HN2-treated recipient amoebae. Other amoebae exposed to lethal doses of UV, or portions cut from them, were combined by microfusion into whole amoebae or amoeba portions lethally treated with HN2. The microsurgical operations were usually done within a day or two after UV-exposure and or HN2-treatment. All experimentally fused amoebae were isolated, as were some of the UV-exposed and HN2-treated control cells. Offspring from isolates were kept together in the same culture. Other controls, i.e., UV-exposed, HN2-treated, and stock (untreated) amoebae were kept in mass cultures.

PROGRESS REPORT

Sensitivity to UV and HN2

The sensitivity of the Colorado strain of *P. carolinensis* to UV at two dose rates is shown in Figure 141. Exposure (12 amoebae per point) to a total dose of 15,000 ergs mm² at 100 ergs mm² sec resulted in 100% lethality. None of the total doses of UV radiation emitted at 30 ergs mm² sec (20 amoebae per

FIG. 141 Sensitivity of *Pelomyxa carolinensis* (Colorado strain) to ultraviolet radiation (254 nm):

* Division of Merck & Co., West Point, Pa.

FIG. 142. Sensitivity of *P. carolinensis* (Colorado strain) to HN2.

point) was as low as those given at the more rapid rate, but doses of 20, 30, and 40 kiloergs mm^2 were lethal to 100% of exposed amoebae, except for a 5% recovery in two 40-kiloergs mm^2 groups (Figure 141). All doses greater than this were consistently lethal to all exposed amoebae.

The sensitivity of this strain to HN2 is shown in Figure 142. We were primarily interested in lethal doses and in death rate. All of the doses shown in Figure 142 were lethal, but the majority of amoebae treated with the lightest dose (0.05 mg/ml) did not die until 3 weeks or longer from the time of treatment. A few of the amoebae in the latter group divided, but all of the offspring eventually died. Most of the amoebae treated with 0.05 mg/ml HN2 appeared to grow abnormally large in size without undergoing division. Amoebae that received the higher doses of HN2 did not divide, and none appeared to increase in size.

Microtransplantation

The microsurgical procedures have been described previously.⁽¹⁾ The volume ratio of unirradiated, untreated stock protoplasm to UV-exposed and HN2-

treated recipients was estimated by visual comparison of donor to recipient cells at magnifications of 40 to 80 diameters. Complete transfer of protoplasm was observed in most of the fusions.

Therapeutic treatment of lethally UV-exposed amoebae by microtransplantation

Amoebae exposed to 45 kiloergs mm^2 at 30 ergs mm^2 sec received protoplasm from unirradiated *P. carolinensis* of the same strain. Seven amoebae were treated in this manner, and each produced a mass culture (Figure 143). All UV-irradiated control amoebae that were not injected with unirradiated donor protoplasm died without cell division. All fusion ratios (volume for volume of protoplasm) of the unirradiated-to-irradiated amoebae were approximately 1:1; in other words, each experimentally fused organism had nearly equal amounts of irradiated and unirradiated protoplasm.

Each fused amoeba was isolated. Cell division was inhibited for about 3 days from the time of UV ex-

FIG. 143—Recovery of supralethally UV irradiated amoebae after injection of unirradiated and untreated donor protoplasm. Failure of supralethally UV irradiated amoebae to recover following injection of HN2 treated protoplasm. N_t = Number of clones; N_0 = number of amoebae at time t .

●, UV irradiated (45,000 ergs mm^2) control amoebae, starting number (N_0) = 33; ○, unirradiated, unfused controls, N_0 = 132; ⊗, UV irradiated (45,000 ergs mm^2) amoebae fused with portions of unirradiated donor amoebae, N_0 = 7; △, UV irradiated (40,000 ergs mm^2) amoebae fused with portions of HN2 treated (0.1 mg/ml) amoebae, N_0 = 7; □, UV irradiated (40,000 ergs mm^2) amoebae fused with portions of HN2-treated (0.2 mg/ml) amoebae, N_0 = 5.

posure (Figure 143). Cell division occurred on the 4th and 5th days, then exceeded the control division rate for about 5 days (Figure 143). Offspring from a single amoeba were kept together as a separate clone, cultured for a month, then discarded.

Therapeutic treatment of lethally HN2-treated (0.125 mg/ml) amoebae by microtransplantation

Untreated stock protoplasm, transplanted into supralethally HN2-treated amoebae, prevented death and promoted cell division as shown in Figure 144. The fusion ratios (volume-for-volume) of untreated stock protoplasm to HN2-treated recipients ranged from 1:15 (one part untreated to 15 parts treated), to 9:1. Forty-seven percent of the fused amoebae had either equal amounts of untreated and HN2-treated protoplasm, or a slight excess of HN2-treated protoplasm. The remainder of the fused amoebae (53%) had an excess of untreated protoplasm, existing primarily in ratios of 2:1 and 3:1. The most rapid recovery was seen in HN2-treated amoebae that received about an equal amount of donor untreated protoplasm. The HN2-treated amoebae that received less than an equal volume of protoplasm

from an untreated donor reached their first cell divisions more slowly. All of the 0.125 mg/ml HN2-treated controls died without dividing (Figure 144). As shown in Figure 142, this dose is 2.5 times the lethal dose.

Lethally UV-exposed amoebae injected with protoplasm from lethally HN2-treated donors

Amoebae that received 50 kiloergs/mm² of UV at 30 ergs/mm²/sec were injected with protoplasm from amoebae lethally treated with HN2 (0.1 and 0.2 mg/ml). Most (70%) of the transfer ratios were 1:1 with approximately equal amounts of UV-exposed and HN2-treated protoplasm in the fused amoeba. None of these fused cells survived longer than HN2-treated controls, or UV-exposed controls (Figures 142 and 143). Furthermore, none of these fused amoebae divided. Thus, protoplasm from HN2-treated amoebae did not promote radiorestitution in the lethally UV-exposed amoebae. Conversely, the protoplasm from amoebae exposed to a lethal dose of UV was ineffective in preventing death in supralethally HN2-treated amoebae.

CONCLUSION

We have explored the UV and HN2 sensitivities of a new strain (Colorado) of *Pelomyxa carolinensis*. Transplantation experiments have shown that recovery is possible after an amoeba has received more than a lethal dose of either UV or HN2. Presumably, death occurred after exposure to these agents because of damage to nuclear DNA. Because of its extensive nature, this damage was not repaired by the cell without proper therapy. Transplantation (injection) of unexposed, untreated protoplasm from donor amoebae replaced the damaged nuclei and, presumably, repaired DNA damage in exposed nuclei. Evidence from the work presented in this report is not adequate to demonstrate DNA repair, but previous investigations of a similar nature on a different species of amoeba showed that the nuclei of amoebae that were exposed to 2.5 times the lethal UV-radiation dose recovered and divided normally after transplantation therapy.⁽⁴⁾

It is clear from the work reported here that UV-radiation irreparably damages restorative agents normally present in unirradiated protoplasm needed by lethally HN2-treated amoebae for recovery, and vice versa. However, supralethally HN2-treated amoeba protoplasm is a very strong restorative agent for supralethally X irradiated amoebae.⁽⁵⁾ Thus, at least in lethally X irradiated amoebae, critical damage causing death can be repaired, or the damaged

FIG. 144. Recovery of supralethally HN2-treated amoebae following injection of unirradiated and untreated donor protoplasm.

N = Number of clones; N_t = number of amoebae at time t .

●, 0.125 mg/ml HN2-treated control amoebae, starting number (N_0) = 40; ○, untreated, unfused controls, N_0 = 132; ⊗, 0.125 mg/ml HN2-treated amoebae fused with untreated amoeba portions, N_0 = 19.

structure replaced, by protoplasm from supralethally HN2-treated donor amoebae. Presumably this is not DNA, because the primary site of damage by nitrogen mustard is in DNA.⁽²⁾ Furthermore, nucleus-free cytoplasm from untreated, unirradiated donor amoebae is restorative.⁽⁵⁾

Lethally X irradiated amoebae are not restored by protoplasm from lethally UV-irradiated donor amoebae.⁽⁴⁾ UV radiation is absorbed by both DNA and RNA, and it would appear from transplantation studies that lethally X irradiated amoebae recover if given protoplasm deficient in DNA, but fail to recover when the protoplasm is deficient in both DNA and RNA. From this, it may be postulated that an RNA species is essential for radiorestitution of supralethally X irradiated cells.

ULTRASTRUCTURE OF OYSTER GAMETES

Edward W. Daniels, Judith M. McNiff, and Arlene C. Longwell¹

PURPOSE AND METHODS

The American oyster (*Crassostrea virginica* Gmelin) is a commercially valuable bivalve mollusk whose sexes normally are separate. Fertilization is external at the time of spawning. This is a report of the ultrastructural anatomy of the oocytes and spermatozoa of this species. Galtsoff⁽¹⁾ wrote a book on this species and presented numerous details of its biology. He described the gametes, published two electron micrographs of oyster sperm cells, but presented no information on the ultrastructure of the oocyte. He discussed the need for additional ultrastructural work on the gametes of this species.

We have studied *C. virginica* sperm cells and oocytes with the electron microscope in an effort to provide a more complete baseline of cytological information on its mature gametes. This information is addressed to future fine-structure studies, research on the genetics, studies of the fertilization and embryological development, and to the potential effects of environmental pollutants on oysters.

MATERIALS AND METHODS

These oysters were shipped in moist plastic containers by air from Milford to Chicago and picked up on arrival. The bivalves were opened, and the go-

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nads were exposed. A biopsy sample was examined in the phase microscope to determine the sex type and quality of gametes. The gametes (sperm or oocytes) were pipetted directly from the moist gonad into 4% glutaraldehyde (Polysciences, Inc., Philadelphia, Pa.) buffered in 0.15 M phosphate (Na_2HPO_4 ; KH_2PO_4) at pH 7.0 in 0.9% NaCl for 2 hr. They were rinsed for several hours or overnight in 3 changes of the saline phosphate buffer and post-fixed in 1% OsO_4 made up in the saline phosphate buffer at pH 7.0. Fixation in osmium was from 1 to 2 hr. The specimens were dehydrated in graded ethanols, and from propylene oxide they were embedded in Epon 812. The blocks were sectioned with a DuPont diamond knife in a Huxley microtome and stained with uranyl acetate (saturated methanol solution) and lead citrate. They were studied in an RCA-EMU 30 electron microscope.

PROGRESS REPORT

Ultrastructure of Oyster Spermatozoa

Typical longitudinal sections through the spermatozoa are shown in Figures 145 and 146. As in other sperm cells, the nucleus is the largest structure in the headpiece and the acrosome is the most anterior, except for the plasmalemma and the occasional vesicle of plasmalemma (bleb) (Figure 145). The axial body⁽¹⁾ (Figure 145, X) is cupped into the anterior portion of the nucleus and capped by the acrosome;

¹ U. S. Department of the Interior, Fish and Wildlife Service, Bureau of Commercial Fisheries, Biological Laboratory, Milford, Connecticut 06460.

FIG. 145. Longitudinal section through center of spermatozoon of the American oyster, *Crassostrea virginica*. A, Aerosome; B, anterior bleb; C, core of axial body; F, filament in proximal portion of tail-piece; L, longitudinal (distal) centriole; M, mitochondrion; N, nucleus; T, transverse (proximal) centriole; X, axial body. 24,000 $\times$.

the axial core⁽¹⁾ (Figure 145, C) lies longitudinally in the middle of the axial body.

The middle piece of the oyster sperm is compressed anterioposteriorly and contains 4 mitochondria, two of which are seen in a typical longitudinal section of the sperm; there are two centrioles. The distal (longitudinal) centriole is continuous with the anterior end of the tail-piece of the sperm (Figures 145 and 146). In our figures, the proximal (transverse) centriole is cut lengthwise (Figure 145), and obliquely (Figure 146).

Filaments in the tail-piece were previously described and demonstrated by Galtsoff⁽¹⁾ as follows: "The tail consists of a pair of axial filaments surrounded by a ring of nine double filaments spaced at equal intervals along the periphery." Figures 145 and 146 of this report show longitudinal sections of these filaments.

FIG. 146. Longitudinal section through a somewhat immature spermatozoon of the American oyster. The section was made at nearly a right angle to that shown in Figure 145. L, Longitudinal (distal) centriole; M, mitochondrion; N, nucleus; P, proximal end of tail piece; T, transverse (proximal) centriole; V, vesicle in the cytoplasm. 46,500 $\times$.

Ultrastructure of Oyster Oocytes

Using the light microscope, Galtsoff⁽¹⁾ described the shape, size, and cytoplasmic inclusions of the mature oocyte of *C. virginica*. Our studies with the electron microscope show the surface of the oocyte to be characterized by small villi on the periphery of the oocyte (Figure 147, VI). A coating of amorphous

FIG. 147. Section through an oocyte of the American oyster. Part of the nucleus (N), nucleolus (NU), and cytoplasm (CY) are shown. The surface of the oocyte is characterized by villi (VI). The cytoplasm contains many ribosomes, minute vesicles, membranes, mitochondria (M), and yolk granules (Y). Some of the latter are surrounded by a double membrane. Nuclear blebs extend into the cytoplasm (NB). Nucleopores can be seen in the nuclear envelope (NE). 11,000.

FIG. 14S. Section of oocyte of *Ameria* in oyster showing branched nuclear bleb (NB), mitochondrion (M), and volk (Y) in the cytoplasm. Mimibodies (MB) are between the two membranes of the nuclear envelope. Nucleoplasm is represented (N). $44,500\times$.

FIG. 149 Oocyte of American oyster showing nucleopore (NP). A mass of ribosomes (R) lies on the cytoplasmic (CY) side of the nucleus (N). Mitochondria (M) and a yolk globule (Y) are also in view. 33,000 $\times$.

material over the oocyte extends from the plasma-lemma to the tips of the villi (Figure 147).

The nucleus of the oocyte is large, 25 to 40 μ according to Galtsoff,⁽¹⁾ and has an acentric nucleolus (Figure 147, NU). Nuclear blebs, some of which are branched, extend into the cytoplasm (Figures 147 and 148, NB). The nuclear envelope (Figure 147, NE) has nucleopores that are demonstrated in tangential (Figure 149, NP) and cross section (Figure 150, NP). A diaphragm may extend across the pores (Figure 150, NP). Numerous ribosomes are shown in the cytoplasm (Figure 149, R) but appear to be excluded from the nucleus by the nuclear envelope.

Mitochondria are demonstrated in the cytoplasm (Figures 147-149, M). Yolk globules (Figures 147-149, Y) are also shown in the cytoplasm. Galtsoff⁽¹⁾ reported the stratification of yolk granules of *C.*

FIG. 150 Side view of nucleopores (NP) in oocyte of the American oyster. Apparently, a diaphragm closes each pore. N, Nucleus, CY, cytoplasm. 30,000 $\times$.

virginica eggs by centrifugation (4,000 times gravity for 10 min). We centrifuged oocytes removed from the gonads of *C. virginica* at 2,500 times gravity for 10 min and obtained some but not complete separation of lipid from protein yolk globules.

CONCLUSION

Preliminary electron microscopic studies have been made on both sperm and oocytes from *Crassostrea virginica*. Some new information has been obtained which can be used in a continuing study of this commercial oyster.

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ENZYMATIC ACTIVITY OF YEAST CELL GHOSTS PRODUCED BY PROTEIN ACTION ON THE MEMBRANES

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PURPOSE AND METHODS

By the action of small basic protein molecules on yeast cells in water suspension at low electrolyte concentration, the membrane is rendered permeable, viability is lost, and cytoplasmic constituents diffuse into the surrounding medium.⁽¹⁾ The cell ghosts obtained in this way retain the cytoplasmic proteins. The washed ghosts were examined for enzymatic activity by adding substrates and observing the enzyme reactions by analyzing the products that diffused into the medium. This procedure appeared particularly important for the study of substrates and coenzymes which do not penetrate into intact cells. The preparation and some of the properties of the ghosts have been described earlier.⁽²⁾ This report deals with their enzymology.

The ghosts were suspended in suitable buffer solutions and mixed with the substrates. After incubation, the suspensions were cleared by centrifugation, and the supernatant fluid was analyzed for the reaction products.

PROGRESS REPORT

A complete survey of yeast enzymes with the present ghosts was beyond realization, and the investigation was restricted to a few examples, mainly from the realm of carbohydrate metabolism. The penetrability of the membrane after protein action is illustrated in Figure 151, which compares the formation of P_i from ATP and PP_i by ghosts with that by intact cells. The supernatant fluid of the ghost preparation was without effect. Glucose-6-phosphate dehydrogenase (Figure 152) is another example that contrasts the usefulness of ghosts with the inefficiency of cells in the metabolism of external substrate and coenzyme. The progress of the reaction, glucose-6-monophosphate + NADP $\xrightarrow{\text{(ghost enzyme)}}$ 6-phosphogluconate + NADPH, was measured by clearing samples by centrifugation, followed by assay of the reduced coenzyme by observing its absorbance at 340 m μ . In a similar experiment (Figure 153) the activity of aconitase and isocitrate dehydrogenase could be demonstrated by incubation of the ghosts with citrate and NADP⁺. Intact cells did not permit the uptake of substrate and coenzyme. Glutamate dehydrogenase also was active in the ghosts.

Other enzyme systems found active in the ghosts included the oxidation of NADH to NAD⁺, hexokinase, the principal amino acid transaminases, uracil riboside hydrolase, β -glucosidase, and L-homocysteine methyltransferase. Multienzyme systems, however, apparently are disrupted in the ghosts. Oxidation of glucose to CO₂ and H₂O was slow and incomplete. This failure may be explained by the extensive intracellular disorganization in the process of converting cells to ghosts which has been demonstrated earlier by electron microscopy.⁽³⁾

FIG. 151. Formation of P_i from ATP and PP_i by ghosts of *Candida utilis*. The substrates (15 mM) were incubated with 150 mg of ghosts per ml. PP_i , +; ATP, ○; ghosts only, △. Experiments with ghosts prepared in (isotonic) 1.0 M mannitol, then transferred into 0.6 M KCl: ATP, ●; ghosts only, ▲. Intact cells with ATP or PP_i , ×.

FIG. 152. Glucose 6-phosphate dehydrogenase activity of yeast cell ghosts. D-glucose-6 monophosphate (3.3 mM) and NADP (3.0 mM) were incubated in 0.02 M potassium phosphate buffer, pH 7.5, with ghosts in a concentration of 16.5 mg, $\square$; 8.3 mg, $\circ$; and 3.3 mg, $\triangle$, per ml. The same experiment with cells, 16.5 mg, $\bullet$, and 8.3 mg, $\blacktriangle$, per ml.

CONCLUSION

The present procedure supplements the earlier methods of yeast enzymology which include sonic and mechanical disintegration of the cell wall, freezing and thawing, plasmolysis with toluene or ethylacetate, or partial autolysis by maceration. In gentleness and efficiency, the present method is not rivaled by any of these procedures. The accessibility of the enzymes in the ghosts suggests their application in all experiments in which substrates and cofactors fail to penetrate into the intact yeast cells.

THE STABILITY OF THE GLYCOSIDIC BOND OF S-ADENOSYLSULFONIUM COMPOUNDS TOWARD ACID

Frederick Schlenk and Cynthia R. Zydek-Crwick

PURPOSE AND METHODS

In experimentation with the biological methyl donor S-adenosylmethionine (Formula I) high stability toward acid was observed.⁽¹⁾ In contrast hereto,

FIG. 153. Aconitase, isocitrate dehydrogenase, and L-glutamate dehydrogenase activity of yeast cell ghosts. Citrate or glutamate (3.3 mM) was incubated with NADP⁺ (3.0 mM) and 33 mg of ghosts per ml of 0.03 M potassium phosphate buffer, pH 7.5. Citrate, $\bullet$; L-glutamate, $\blacktriangle$; substrate omitted, $\triangle$; ghosts only, $\times$; intact cells with citrate and NADP⁺, $\circ$; intact cells only, $+$.

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the demethylated compound, S-adenosylhomocysteine (Formula II) was found to be labile at the glycosidic bond to the same degree as other biological adenosine compounds, including AMP, ADP, ATP, DPN, and TPN. A systematic exploration of this phenomenon

Formulas I and II. S-adenosylmethionine and S-adenosylhomocysteine. The principal sites of acid hydrolysis are indicated by letters.

appeared to be of interest in relation to nucleoside chemistry, and practical procedures for the separation of S-adenosylhomocysteine from S-adenosylmethionine were indicated. The acid resistance of the sulfonium compound was studied in detail, and the products obtained were analyzed.

PROGRESS REPORT

The hydrolysis of the glycosidic bond of S-adenosylmethionine (Formula I, bond A) and of the corresponding linkage in S-adenosylhomocysteine (Formula II) was determined by chromatographic assay

Fig. 154.—Acid hydrolysis of S-adenosylmethionine and S-adenosylhomocysteine. The samples of S-adenosylmethionine (5 mM) in 1.0 N HCl (○) and in 0.1 N HCl (●) were heated at 100°. The decrease in the concentration of the sulfonium compound and the coincident formation of adenine were determined by ion exchange chromatography and spectrophotometry. S-adenosylhomocysteine (5 mM) was hydrolyzed in the same way in 1.0 N HCl (△) and in 0.1 N HCl (▲). The residual S-adenosylhomocysteine was determined by deamination and spectrophotometry.⁽²⁾

of the adenine which is formed in the process (Figure 154). It may be seen that the reaction constants for the hydrolysis of these compounds in 1.0 N HCl differ by two orders of magnitude. In 0.1 N HCl, the difference is much less, which suggested that the hydrolysis takes a different course. By paper chromatography and assay with adenosine deaminase⁽²⁾ it was found that in dilute acid, scission C (Formula I) is the first step; this leads to 5'-methylthioadenosine, which undergoes scission A very rapidly. In 2 N and 6 N HCl, bond B (Formula I) is broken in preference to bond C.

The difference between S-adenosylmethionine and S-adenosylhomocysteine in their sensitivity toward acid can be used to advantage for selective destruction of S-adenosylhomocysteine in acid extracts of cell material. S-adenosylmethionine preparations are sometimes contaminated by up to 20% of S-adenosylhomocysteine, and the elution peaks of the two compounds from Dowex 50 H⁺ columns usually merge or overlap. S-adenosylhomocysteine could be destroyed by heating the initial 1.5 N perchloric acid extract of yeast by heating to 100° for 5 min. The loss of S-adenosylmethionine was less than 5%, while S-adenosylhomocysteine was reduced to an insignificant level. Figure 155 shows the chromatographic analysis. As a control the same experiment was performed without heating of the extract from another sample of the same yeast.

Fig. 155.—Ion exchange chromatography of adenine, S-adenosylhomocysteine, and S-adenosylmethionine in yeast extract. A Dowex 50 H⁺ column was used. Nucleosides and nucleotides appeared in the early fractions, followed by adenine (peak I) and a mixture of S-adenosylhomocysteine and S-adenosylmethionine (peak II), dotted line. The same experiment with previously heated extract (5 min, 100°) is represented by the solid line; only S-adenosylmethionine was found under these circumstances in peak II.

In the latter, S-adenosylhomocysteine preceded S-adenosylmethionine and merged with it in the elution as judged by paper chromatography and enzymatic analysis.⁽²⁾

CONCLUSION

The stabilizing effect of the sulfonium group on the glycosidic bond is of great importance. Current concepts of the acid hydrolysis of nucleosides suggest that a proton is attached initially to nitrogen atom 3 or 7 of the purine and then transferred to the carbohydrate to open the furanoid ring. Difficulty in the initial attachment of the proton or in its transfer to the carbohydrate moiety may account for sluggish hydrolysis. The purine moiety of S-adenosylmethionine should offer the same chance for protonation as

in other purine nucleosides. However, the proximity of the sulfonium pole to the purine ring may be inhibitory. As an alternative, the sulfonium group may stabilize the furanoid ring and thus prevent transfer of the proton to the ring oxygen. Perhaps a combination of both effects is responsible for the low rate of acid hydrolysis of the glycosidic bond of S-adenosylmethionine.

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THE SPECIFICITY OF S-ADENOSYL-L-METHIONINE SULFONIUM STEREOISOMERS IN SOME ENZYME SYSTEMS

Vincenzo Zappia and Fritz Schlenk

PURPOSE AND METHODS

Enzymes usually show high specificity toward the D- or L-form of compounds containing an asymmetric carbon atom, particularly if the action involves the center of asymmetry. Similar stereoisomerism is observed at the sulfur atom of sulfonium compounds:

On the basis of polarimetry, the two stereoisomers are designated as (+) and (−) sulfonium form, because the absolute configurations have not yet been elucidated,⁽¹⁾ and no basis exists for the relation to the D- and L-series of carbon stereoisomers. There has been only one investigation to this date which deals with the enzymatic specificity of sulfonium stereoisomers, (+)S-adenosyl-L-methionine and (−)S-adenosyl-L-methionine; only the latter was found active in enzyme systems.⁽²⁾ It appeared rewarding to explore this stereoisomerism in more detail. For this, (±)S-adenosyl-L-methionine was synthesized by methylation of S-adenosyl-L-homocysteine; the activity of the racemic form was compared with that of biosynthetic, natural (−)S-adenosyl-L-methionine in several enzyme systems. For analytical convenience, all methyl

donors were ¹⁴CH₃-labeled; the material for the decarboxylation experiments was ¹⁴COOH-labeled.

PROGRESS REPORT

For the tests, histamine N-methyltransferase, acetylserotonin methyltransferase, L-homocysteine methyltransferase, and S-adenosyl-L-methionine decarboxylase were employed. In the methylation of histamine (Figure 156) twice as much of the synthetic (±)sulfonium compound was needed as of the biological (−)form to achieve identical speed of the reaction. Inactivity of the (+)stereoisomer is indicated hereby. The same observation was made with the acetylserotonin methyltransferase system. In contrast to this specificity, both stereoisomers were found active in the methylation of L-homocysteine to L-methionine (Figure 157). The transmethylation with the (±)stereoisomer went beyond the critical value of 50%, which shows that the (+)form is active. The higher speed of reaction with the racemic methyl donor as compared with the (−)form indicates that the (+)stereoisomer reacts faster than the biological (−)form.

In the decarboxylation of S-adenosyl-L-methionine to S-adenosyl-(5')-3-methylthiopropylamine (Figure 158), only the biosynthetic (−)sulfonium compound reacted. The progress of the reaction with the racemic compound was virtually the same as that observed with half the concentration of the (−)form; the de-

FIG. 156.— Sulfonium stereoisomers of S-adenosyl-L-methionine as methyl donors in the methylation of histamine.⁽³⁾ Purified enzyme was incubated with 0.2 μ mole of histamine and 50 ($\square$) or 25 ($\blacksquare$) μ moles of (—)S-adenosyl-L-($^{14}\text{C}_3$)-methionine, or 50 μ moles ($\blacktriangle$) of ($\pm$)S-adenosyl-L-($^{14}\text{C}_3$)-methionine.

FIG. 157. The methylation of L-homocysteine by sulfonium stereoisomers of S-adenosyl-L-methionine. The enzyme was incubated⁽⁴⁾ with 2.5 mM L-homocysteine and 1.0 M (—)S-adenosyl-L-($^{14}\text{C}_3$)methionine ($\bullet$), or 1.0 M ($\pm$)S-adenosyl-L-($^{14}\text{C}_3$)methionine ($\circ$).

FIG. 158.— Decarboxylation of the sulfonium stereoisomers of S-adenosyl-L-methionine. The decarboxylase from *Escherichia coli* was used⁽⁴⁾ for incubation with ^{14}C -carboxyl-labeled (—)S-adenosyl-L-methionine, 1.05 mM ($\blacktriangle$); 0.525 mM ($\triangle$); under the same conditions, ^{14}C -carboxyl-labeled ($\pm$)S-adenosyl-L-methionine was tested, 1.05 mM ($\circ$).

carboxylation of the racemic product did not exceed 50%.

CONCLUSIONS

The activity of both sulfonium stereoisomers of S-adenosyl-L-methionine in the L-homocysteine methyltransferase system is an exception to the usual requirement for the (—)sulfonium donor in transmethylation. The biological implications of this remain to be explored. The sulfonium stereospecificity of propylamino group transfer from S-adenosyl-(5')-3-methylthiopropylamine in the formation of spermidine and spermine from putrescine has not yet been tested. However, the present observation that only (—)S-adenosyl-L-methionine is decarboxylated, makes it improbable that the (+)propylaminosulfonium compound plays a part in this process.

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THE METABOLISM OF MITOCHONDRIAL PROTEINS

Robert W. Strick* and Carl Peraino

PURPOSE AND METHODS

An elucidation of the pattern of turnover of the various protein components of the mitochondria should lead to a better understanding of the normal dynamics of this organelle. Fletcher and Sanadi⁽¹⁾ estimated the renewal rate of liver mitochondria from the kinetics of the decay in radioactivity of a labeled amino acid with no correction for the possible effects of reutilization of the isotope. Because the rate of decay in activity in the lipid fraction labeled with acetate was similar, they suggested that the mitochondria are renewed as entities with a half-life of about 10 days. Subsequent studies by a number of investigators have tended to confirm an 8- to 10-day half-life for a number of crude mitochondrial protein fractions.

In the present study, rats were exposed continuously to $^{14}\text{C}\text{O}_2$ through the addition of $\text{Ca}^{14}\text{C}\text{O}_3$ to the diet, which was then fed fractionally 24 times a day. The turnover rates of the various liver protein fractions were calculated from the specific radioactivities of the guanidine carbon of protein arginine and of excreted urea after various periods of exposure.⁽²⁾ Mitochondria were separated into six operational fractions, all of which appeared to have about the same turnover rate: 0.13 to 0.16 days⁻¹.⁽³⁾ The turnover rates were also estimated from enzyme activity kinetics by assaying alanine aminotransferase⁽⁴⁾ and ornithine ketoacid aminotransferase⁽⁵⁾ at intervals after changing the rats from a normal diet to a diet high in protein (and the reverse) or after the injection of the corticosteroid, prednisolone. In contrast to the results obtained from isotope incorporation, the renewal rates of the enzymes were very rapid in all cases: 0.67 to 0.95 days⁻¹.⁽³⁾ Additional experiments have been carried out in an attempt to resolve this difference.

PROGRESS REPORT

The last and most significant step in the study of the metabolic turnover of mitochondrial proteins is the direct measurement of the renewal of a pure protein from this particle. Ornithine aminotransferase was chosen because of our experience with it, the ease of its purification,⁽⁶⁾ and the availability of estimates of its turnover from the kinetics of its induction.⁽³⁾ Confidence in the validity of the latter method would

be enhanced if similar values could be obtained from isotope incorporation experiments.

We first attempted to isolate ornithine aminotransferase from rat liver mitochondria with the aid of the antibody to the enzyme, which had been prepared earlier.⁽⁷⁾ An unacceptably large amount of non-specific protein was, however, always precipitated with the enzyme-antibody complex, rendering the technique useless for this purpose. Next, we attempted to isolate the enzyme from a single liver after the addition of about a 10-fold excess of pure, carrier ornithine aminotransferase. While this procedure provided sufficient quantities of protein for isotope analysis, the additional error introduced by the isotope dilution made interpretation of the results difficult. Finally, the purification technique was perfected to the point that amounts of ornithine aminotransferase sufficient for isotope analysis could be isolated directly from the livers of as few as 4 rats (2 rats, when induced). Although these experiments have been fraught with methodological difficulties, acceptable results were obtained in one experiment. Table 72 gives a comparison of the renewal rate of ornithine aminotransferase as determined by enzyme induction and isotope incorporation. The values agree quite well. The renewal rates of the other (nonenzyme) fractions are shown for comparison and are similar to those obtained for such fractions previously.⁽³⁾ Thus, there seems to be no doubt that this enzyme is renewed rapidly in the mitochondria and at a rate about 5 times greater than that of the other protein fractions.

It has been shown that there is a limited but significant synthesis of protein in the mitochondrion (see Reference 8); however, it has also been shown that most of this protein is associated with the inner membrane.⁽⁹⁾ Because ornithine aminotransferase is easily solubilized, it is undoubtedly a matrix enzyme and its

TABLE 72. TURNOVER RATES OF ORNITHINE AMINOTRANSFERASE AND OTHER MITOCHONDRIAL FRACTIONS

Component	<i>k</i> in days ⁻¹	
	Enzyme induction	Isotope incorporation
Ornithine aminotransferase	0.89	1.09
"Structural protein"		0.15
Residue		0.16
pH 5 precipitate		0.13
55 precipitate		0.12

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TABLE 74. INCORPORATION OF ^{14}C -LEUCINE INTO MITOCHONDRIA INCUBATED *in Vitro*

Component	Specific activity in cpm/mg protein	
	KCN; no ATP	+ATP
"Structural protein"	152	1100
Residue	72	121
55° precipitate	18	32
Ornithine aminotransferase	4	2

synthesis *in situ* is unlikely. Its very rapid renewal rate, the speed of its induction, and the speed of its disappearance after removal of an inducing agent could be explained, however, by the *in situ* synthesis of the enzyme. To test this possibility, mitochondria were incubated with ^{14}C -leucine and the other components known to be required for *in vitro* protein synthesis.⁽⁸⁾ As a control, a parallel incubation was carried out under conditions which preclude protein synthesis, i.e., no ATP was added, while CN^- was included. The results are given in Table 74. It would seem that while there was incorporation of isotope into the "structural proteins" of the mitochondria under these conditions, no synthesis of ornithine aminotransferase occurred.

CONCLUSION

Ornithine aminotransferase has been shown both by isotope incorporation and the kinetics of enzyme induction to have a renewal rate of about once per day, a rate 5 times as rapid as that for other mitochondrial proteins. This rapid rate apparently is independent of mitochondrial generation; this independence might be explained by the *in situ* synthesis of ornithine

aminotransferase. Under the conditions employed in these experiments, however, no labeled amino acid was incorporated into ornithine aminotransferase *in vitro* although significant amounts of isotope were incorporated into the "structural protein." Our previous conclusions are confirmed that the mitochondria are in a dynamic state and alteration of the enzyme composition does not depend on the formation of new mitochondria.

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THE HETEROENZYMES OF ORNITHINE AMINOTRANSFERASE

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PURPOSE AND METHODS

Enzymes from different sources which catalyze the same reaction and have similar substrate specificities and kinetic properties are termed *heteroenzymes*. Heteroenzymes almost certainly have similar arrangements of amino acids about the catalytic site, but their amino acid sequence in other parts of the mole-

cule may differ, resulting in differences in conformation, electrophoretic mobility, solubility characteristics, and immunological properties.

The purpose of this project was to characterize a number of the heteroenzymes of ornithine aminotransferase through a comparison of their electrophoretic and immunological properties. The heteroenzymes were prepared from mitochondria isolated from the tissues of a large number of species of rodents and other animals available in this Division, using the

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first four steps of the procedure described by Peraino, Bunville, and Tahmisian.⁽¹⁾ Because we started with mitochondria, the specific activity of ornithine aminotransferase was quite high at this point. Electrophoretic mobility is of interest because of the implications regarding the structural relationships, both three-dimensional and chemical; it was investigated using disc electrophoresis. After migration, the gel plugs were cut into 4-mm lengths and assayed for the enzyme. The comparative immunology of the heteroenzymes is of interest because of the implications regarding the taxonomic relationships of the sources of the enzyme. The immunological cross reactivity of the heteroenzymes was studied by titration of the enzymes with antisera obtained from rabbits which had been injected with pure rat liver ornithine aminotransferase. Inhibition of the enzyme activity by the antibody was also investigated.

PROGRESS REPORT

An analysis of the activity of each preparation revealed a wide spread in the amount of enzyme among the different species (Table 75). The activity of ornithine aminotransferase was expressed in units per gram of tissue; the limit of sensitivity was 0.05 units/g. *Peromyscus leucopus* had the highest level of enzyme in the livers examined with 0.72 units/g. Species of the families Cricetidae and Muridae also had high levels of enzyme, and *Gallus gallus* was just below these amounts. Although the amount of enzyme in *Canis familiaris* liver and kidney, *Bos taurus* kidney,

TABLE 75. AMOUNT OF ORNITHINE AMINOTRANSFERASE^(a)

Organism	Organ	Ornithine aminotransferase, units/g tissue
<i>Peromyscus leucopus</i>	Liver	0.72
<i>Peromyscus californicus</i>	"	0.45
<i>Rattus norvegicus</i>	"	0.36
<i>Oryzomys palustris</i>	"	0.16
<i>Rattus rattus</i>	"	0.13
<i>Microtus pennsylvanicus</i>	"	0.10
<i>Mus musculus</i>	"	0.09
<i>Meriones</i>	Kidney	0.08
<i>Gallus gallus</i>	Liver	0.08
<i>Sigmodon hispidus</i>	Liver	0.06
<i>Canis familiaris</i>	Liver	<0.05
<i>Canis familiaris</i>	Kidney	<0.05

^(a) The following species were found to have an amount of ornithine aminotransferase significantly less than 0.05 units/g tissue: *Bos taurus* liver and kidney, *Citellus tridecemlineatus* liver and kidney, *Cavia porcellus* liver and kidney, *Mus musculus* kidney, *Sylvilagus* liver, *Mastomys natalensis* liver, *Meriones* liver, *Perognathus pecillatus* liver and kidney, *Microtus pennsylvanicus* kidney, and *Rana pipiens* liver.

TABLE 76. EQUIVALENCE OF ANTISERUM AND ORNITHINE AMINOTRANSFERASE

Organism	Organ	Calculated, units/0.01 ml ^(a)	Slope = 1, units/0.01 ml ^(a)
<i>Rattus norvegicus</i>	Liver	0.076	0.078
Ascites tumor cells		0.070	0.074
<i>Rattus rattus</i>	Liver	0.064	0.064
<i>Mus musculus</i>	Liver	0.063	0.076
<i>Peromyscus californicus</i>	Liver	0.055	0.055
<i>Peromyscus leucopus</i>	Liver	0.05	0.05
<i>Meriones</i>	Kidney	0.03	0.05
<i>Canis familiaris</i>	Liver	0.032	0.026
<i>Bos taurus</i>	Kidney	0.028	0.031
<i>Gallus gallus</i>	Liver	0.00	0.00

^(a) Volume of antiserum diluted 1:3.

Mastomys natalensis liver, and *Citellus tridecemlineatus* liver was low, concentration of the preparations made possible characterization of these enzymes.

The heteroenzymes of ornithine aminotransferase showed very similar electrophoretic mobilities. Purified *Rattus norvegicus* liver and kidney ornithine aminotransferase and the partially purified heteroenzyme preparations moved about 32 mm under the conditions chosen. Enzyme activity was usually found in two adjacent sections of the gel, although it sometimes ranged from 1 to 5 sections of the gel. When the purified samples were stained, a leading band of enzyme was well defined, but a trailing edge diffused to clearness. This was not surprising because the enzyme is known to aggregate.⁽¹⁾ A stained protein band, however, did not always correspond to the sections showing enzyme activity; apparently, the amount of protein at the site of enzyme activity was inadequate to produce a visible stain.

The immunological titration of the enzyme preparations was next performed to obtain a quantitative measure of the cross reactivity of the heteroenzymes. When equivalence was calculated, some of the slopes varied slightly from the theoretical value of 1. Thus, equivalence was estimated both from the calculated slope as well as the theoretical slope (Table 76). A comparison of equivalence showed that 4 groups were formed (Figure 159). Group 1, which consisted of those species in the family Muridae, i.e., *Rattus norvegicus*, *Rattus rattus*, *Mus musculus*, and ascites tumor cells, reacted with the highest equivalence (0.07 units/0.01 ml antiserum). Group 2, which was from the closely related family Cricetidae, i.e., *Peromyscus californicus*, *Peromyscus leucopus*, and *Meriones* had a lower equivalence point (0.05 units/0.01 ml antiserum). The animals from a different order formed group 3, i.e., *Canis familiaris* and *Bos taurus*, and

these had equivalence at a still lower value (0.03 units/0.01 ml antiserum). *Gallus gallus*, which is in a different class, formed group 4. This enzyme was either nonreactive, or the extent of reactivity was too low to measure. Thus, it is readily seen that equivalence depended on the taxonomic relationship of the different species.

The inhibition of *Rattus norvegicus* liver ornithine aminotransferase by its antiserum was determined. Various amounts of antiserum were incubated overnight at 0° to 4°C with a constant amount of enzyme. The samples were stirred, and an aliquot was removed and assayed for enzyme activity. The remaining sample was centrifuged, and the supernatant solution was assayed. The amount of anti-serum added plotted against the enzyme units recovered before centrifugation resulted in a two component curve (Figure 160) which was resolved by computer analysis. The first component represented the inactivation of the enzyme by small amounts of antibody. The suspension was a mixture of free enzyme and antibody-enzyme complex. Complete association of the enzyme with the antibody occurred about at the breakpoint of the curve, as confirmed by the lower curve which shows that all of the enzyme activity was sedimentable at that point. The second component represented the further inhibition of the antibody-enzyme complex by larger amounts of antibody. Extrapolation of this line to the ordinate indicated that the antibody-enzyme complex was 67% as active as the free enzyme. The antibody-enzyme complex was also inhibited by

FIG. 159.—Disproportionality of cross reactivity. Units of ornithine aminotransferase (OT) added 0.01 ml antiserum plotted against units of enzyme in supernatant solution after incubation 0.01 ml antiserum. +, *Rattus norvegicus* liver; Δ, *Peromyscus californicus* liver; ○, *Canis familiaris* liver; ●, *Gallus gallus* liver.

FIG. 160.—Ornithine aminotransferase activity remaining after incubation with various amounts of antiserum. Δ, after incubation but before centrifugation; ○, after incubation and after centrifugation.

increasing amounts of antibody: each 0.1 ml of antibody removed 0.107 units of ornithine aminotransferase. This provides evidence that the antibody does not react with the catalytic site, and that the inhibition produced was probably caused by allosteric effects.

CONCLUSION

Electrophoretic characterization is based on the net charge of the protein. The internal groups of the protein hold the conformation of the molecule; the external groups, however, determine the net charge, because they are not involved with other charged groups. The electrophoretic mobility of all of the preparations was similar. Thus, apparently all of the enzymes, regardless of source, are quite similar in the amino acids which affect the charge on the molecule.

Immunochemical techniques are used as a basis for taxonomic classification. The antiserum to *Rattus norvegicus* liver ornithine aminotransferase in this study reacted disproportionately with the heteroenzymes of ornithine aminotransferase. Thus, it is readily seen that the greater the taxonomic difference, the lesser the equivalence value between antiserum and enzyme.

The extent of the cross-reactivity of ornithine

aminotransferase is significant, however. Because the determinants are composed of small chemical groups in a specific arrangement, both the groups and the conformation of the heteroenzymes at the determining sites must be similar. Apparently the *Gallus gallus* enzyme is significantly different in either one or both respects, because there was no cross reaction.

Further characterization will involve the study of

the kinetics and substrate specificities of the heteroenzymes and their precipitation and inhibition by the antisera.

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THE HOMOGENEOUS DISTRIBUTION OF MEMBRANE-BOUND AND SOLUBLE MITOCHONDRIAL ENZYMES*

Robert W. Swick,[†] Sandra L. Tollaksen, Sharon L. Nance and John F. Thomson

Mitochondria were isolated from normal rat liver, kidney, and heart and from mouse liver and ascites tumor cells. The mitochondria were then distributed through a linear sucrose density gradient in a zonal centrifuge. The distributions of the activities of several enzymes known to be associated with either the outer or inner membranes were compared with the distributions of the soluble mitochondrial enzymes. The midpoints of the distributions appeared to be significantly different when the two classes of enzymes were plotted against the mean particle diameters of the fractions as recovered. On the other hand, when the distributions of the outer membrane enzymes were calculated as a function of the area of the particles

and the distribution of the soluble enzymes was expressed as a function of the volume of the mitochondria, the particle diameters corresponding to the respective midpoints were quite similar. This congruence suggests that mitochondria are, after all, homogeneous with respect to the enzyme activities examined. Furthermore, the distributions of the inner membrane activities were like those of the outer membrane enzymes. If the enzyme composition of the membrane is homogeneous, the area of the inner membrane appears to be proportional, not to the volume of the mitochondria, but to the area of the outer membrane.

The excellent congruence of the distributions of rotenone insensitive NAD-cytochrome *c* reductase and other membrane-bound enzymes is strong evidence that the reductase is a normal constituent of one of the mitochondrial membrane systems.

* Abstract of paper in preparation.

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THE SEDIMENTATION COEFFICIENT OF ORNITHINE AMINOTRANSFERASE

Lyle G. Bunville

PURPOSE AND METHODS

The apparent molecular weight of ornithine aminotransferase, as determined by equilibrium ultracentrifugation, has been shown to vary from 132,000 in very dilute solutions up to approximately 200,000 at concentrations of the order of 0.3 mg/ml.^(1,2) From information available at that time it could not be ascertained whether this variation in molecular weight was due to the presence of noninteracting species (heterogeneity), or a consequence of the association of the enzyme. In order to establish the origin of the concentration dependence of the apparent molecular weight, particular emphasis has been placed upon its

sedimentation behavior, utilizing the technique of band sedimentation.⁽³⁾ In this technique, a thin lamella of the enzyme solution is layered onto a denser sedimentation solvent in an ultracentrifuge cell. The subsequent transport of the narrow band of enzyme macromolecules through this solvent is observed spectrophotometrically as a function of time. The principle advantages of this technique are 1) extreme economy, 2) sensitivity to heterogeneity, and 3) adaptability to treatment of the enzyme with a wide variety of reagents. In the latter instance, sedimentation of the enzyme in a solvent containing the substrate for the enzyme allows the use of enzyme con-

TABLE 77. CONCENTRATION DEPENDENCE OF THE SEDIMENTATION COEFFICIENT OF ORNITHINE AMINOTRANSFERASE

Initial conc., mg/ml	$s_{25,w}$, S
0.020 ^(a)	5.35
0.038 ^(a)	5.50
0.052 ^(a)	5.45
0.26	8.43
0.51	9.09
1.0	9.78
2.1	10.0
6.3	10.4

^(a) Sedimentation in presence of substrate.

centrations an order of magnitude lower than feasible when the enzyme is observed directly.⁽⁴⁾

PROGRESS REPORT

As seen from the representative results presented in Table 77, the sedimentation coefficient, $s_{25,w}$ (corrected to a solvent viscosity and density corresponding to water at 25°C), of ornithine aminotransferase is strongly dependent upon the concentration of the enzyme.

By combining the sedimentation coefficient of 5.43 S and molecular weight of 132,000 obtained at extreme dilution, it may be estimated that the molecular weight corresponding to a sedimentation coefficient of 10.4 S is approximately 350×10^3 .

From the sedimentation profiles of the enzyme at high concentrations, it may be inferred that the high molecular weight species is substantially asymmetric in shape and that the rate constant for its dissociation is small.

CONCLUSION

From an investigation of the concentration dependence of the sedimentation coefficient of ornithine aminotransferase, it has been concluded that the variation of apparent molecular weight with concentration⁽²⁾ is the result of the self-association of the enzyme.

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ULTRAVIOLET MICROSCOPY OF EMBRYONATING TOXASCARIS LEONINA OVA

George Srihla

PURPOSE AND METHODS

Cursory examination of an occasional nematode or rotifer during the course of ultraviolet microscopy studies in the last decade has revealed that the distribution and concentration of materials which absorb monochromatic energy in these organisms seems to differ from what is observed in yeast,⁽¹⁻⁴⁾ protozoan,^(5,6) or mammalian cells. *Toxascaris leonina* (Ascaridae) eggs isolated from feces of Argonne beagles, or from adult worms obtained by treatment of the dogs with piperazine hydrochloride, furnished an opportunity to examine the absorption pattern of embryonating eggs of this species. The eggs had been obtained in an investigation, with Dr. Calvin Poole, of the incidence and identity of ascarids in beagles in the Argonne colony by Laura Hardester, a visiting faculty investigator.

The nematode egg is notoriously resistant to chemicals used as killing and fixing agents in conventional and electron microscopy. A method that would permit study of the living organism and yield information about certain biochemical processes of development of the embryo seemed worth investigating. At the very least it would provide information about the location and concentration of materials absorbing at specific wavelengths that would be useful for studies of UV-irradiation effects. Leads to information useful for the elimination or control of *T. leonina* as a specific parasite, or nematodes in general, could be of considerable economic importance.

Embryonating ova in various stages of development, mounted in water on quartz slides under quartz covers, were photographed at 297 and 265 nm. A 32× Ultrafluor objective was used in conjunction with apparatus already described.⁽⁷⁾

FIGS. 161 and 162. Ultraviolet micrographs of stages in the development of the larva of *Toxascaris leonina*, a parasitic nematode. The paired micrographs were made at 265 nm (left) and 297 nm (right).

PROGRESS REPORT

Ultraviolet micrographs of embryonating *T. leonina* eggs made at 297 and 265 nm revealed strong absorption at 265 nm (Figures 161 and 162). The absorption pattern is similar to that observed in cysts of *Entomocha invadens* just before encystation;⁽⁵⁾ the ultraviolet-absorbing material is uniformly distributed throughout the cytoplasm of the cells rather than concentrated in the nucleus, as, for example, is common for mammalian cells, or in the chromatoid body of *E. invadens* cysts during early stages of encystation. While the chromatoid body of *E. invadens* was shown by electron microscopy to be an aggregation of ribosome-like particles that dispersed in the mature cysts, the UV-absorbing material of *T. leonina* eggs has not been identified. Absorption at 265 nm and location in the cytoplasm suggests that it may be ribonucleoprotein.

Nematodes belong to the phylum Aschelminthes,

which includes the rotifers, and have a "fixed" somatic cell number reached at about the first larval stage. No cell division takes place later than their early embryology. Until hatching, there is no uptake of material from the environment (except gaseous). After hatching, growth is by increase in cell size. Because of these peculiarities, the distribution of the UV-absorbing material during cell division was of particular interest. The series of ultraviolet micrographs (Figures 161 and 162) show that the UV-absorbing material present in the one-celled stage is shared about equally at each cell division. Unfortunately, the size and movement of the larval stage has interfered with observation of the distribution of the UV-absorbing material during differentiation.

CONCLUSION

The embryonating ova of *T. leonina* contain an abundance of a more or less homogeneously dis-

tributed ultraviolet-absorbing (265 nm) material. Since absorption of energy is necessary for an effect, the eggs of *T. leonina* should be interesting material for ultraviolet irradiation studies. Some time-lapse motion picture studies of the effects of irradiation with 253.7 nm energy on dividing eggs and on larvae have been initiated.

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ULTRAVIOLET MICROGRAPHY OF PENETRATION OF EXTRANEIOUS CYTOCHROME C INTO THE YEAST CELL*

George Svihla, Julia L. Dainko, and Fritz Schlenk

Candida utilis and *Saccharomyces cerevisiae* in water suspension were found to be very sensitive to exogenous cytochrome c. The protein was taken up by the cells, and the viable count was reduced to a few percent of the initial value. Micrography at 405 nm revealed penetration of cytochrome c into the

* Abstract of a paper published in *J. Bacteriol.* **100**, 498-504 (1969).

interior of the cell. The cytoplasmic membrane lost its capacity to retain intracellular constituents; ultraviolet-absorbing compounds were released into the medium. When budding cells were subjected to treatment with cytochrome c, the mother cells were found more susceptible than the buds. Phosphate buffer protected the cells and spheroplasts against cytochrome c.

ESTIMATION OF COMPONENT SIZE AND SEPARATION IN FUSED GAUSSIAN DISTRIBUTIONS

Peter D. Klein

PURPOSE AND METHODS

It is of general interest to be able to detect the presence of two components in a distribution when these components are not visibly resolved in the enveloping peak form. Previous work in this area⁽¹⁾ achieved the resolution of multiplet gaussian distributions when it was assumed that each component was identical in size and dispersion to the others. As useful as this is, it would be helpful if it could be applied to components of unequal size. Using the computer-generated distributions employed in previous studies,⁽²⁻⁴⁾ I have examined the higher moments of the compound distribution and found a relationship between these moments, the relative sizes of two unequal components and the separation between them.

PROGRESS REPORT

Two populations, n_1 and n_2 , having the same dispersion σ , but whose means are separated by an increment, ΔM , are combined, interval by interval, to give a compound peak with mean M , and dispersion σ . The moments from first to sixth are computed as shown in Table 78, which also lists their accepted statistical significance.

A series of populations ranging from proportions of 5:95 to 95:5, and separated by distances of $\Delta M/\sigma$ from 0.8 to 2.6 were synthesized, combined, and the higher standard moments generated from the compound distribution. Examination of the values for these standard moments showed a relationship between the fifth and sixth moments, as illustrated in Figure 163. In each compound peak whose com-

TABLE 78. DESIGNATIONS OF HIGHER MOMENTS OF GAUSSIAN DISTRIBUTIONS

Moment:	$\Sigma (\mu - x)^n$
Standard moment:	$\Sigma (\mu - x)^n / [\Sigma (\mu - x)^2]^{n/2}$
n	Parameter
1	Mean
2	Dispersion
3	Skewness
4	Kurtosis
5	Undesignated
6	Undesignated

fifth and sixth moment uniquely characterise the composition of a compound peak.

The coefficients of the two intersecting series of parabolas were evaluated and their relationship to $\Delta M/\sigma$ and n_1/n_2 was determined. The separation between components could be expressed by the equation

$$\Delta M/\sigma = \frac{A_{01}}{A_{21}} = 1.9231 - 4.9053, \quad (1)$$

where A_{21} is obtained from the fifth and sixth standard moments (x, y) from the relationship

$$A_{21} = (y - 5.2308) (4.1517 + x^2), \quad (2)$$

and A_{01} is given by

$$A_{01} = 4.1517A_2 + 5.2308, \quad (3)$$

The proportion n_1/n_2 was obtained from the somewhat more complicated expression

$$n_1/n_2 = \frac{\log [13.93(0.500 - A_{22})] + 1.5097}{2.756}, \quad (4)$$

where

$$A_{22} = (y - 13.132) x^2. \quad (5)$$

It is possible to determine the separation and composition of two components from these equations, relying solely upon the fifth and sixth standard moments of the distribution. How well these equations will determine these parameters is shown in Tables 79 and 80. Here, the actual moments of the pure, synthetic distributions were re-inserted in the equations derived, and both parameters were computed over the entire range studied. Certain limitations are evident in the two tables; for example, the determination of component size is probably unreliable when the separation between peaks is low and the components are of grossly different proportions. At separation

TABLE 79. FITTING OF COMPONENT SIZE AS A FUNCTION OF PEAK SEPARATION AND COMPONENT SIZE

n_1	0.100	0.150	0.200	0.250	0.300	0.350	0.400	0.450
M/σ	n_2 found							
1.00	0.275	0.292	0.316	0.344	0.374	0.409	0.452	0.511
1.20	0.163	0.211	0.259	0.300	0.356	0.393	0.438	0.501
1.40	0.131	0.200	0.248	0.289	0.333	0.373	0.422	0.488
1.60	0.026	0.159	0.220	0.269	0.323	0.367	0.418	0.485
1.80	0.085	0.168	0.221	0.267	0.312	0.358	0.410	0.479
2.00	0.079	0.158	0.210	0.258	0.310	0.357	0.410	0.479
2.20	0.112	0.167	0.213	0.258	0.305	0.353	0.407	0.477
2.40	0.119	0.165	0.209	0.255	0.306	0.354	0.409	0.479
2.60	0.136	0.171	0.211	0.256	0.303	0.353	0.410	0.479

FIG. 163. Fifth and sixth standard moments of compound peaks grouped by peak separation and component size.

ponents were separated by the same $\Delta M/\sigma$, the values for the fifth and sixth standard moments formed a parabola that was concave upward from the origin and symmetrical about the axis. The value of the fifth moment increased in proportion to the imbalance of the two component populations, and parabolas representing larger and larger values of $\Delta M/\sigma$ were concentric in shape.

Through the same points, one can also draw a series of parabolas which begin as narrow cones, concave downwards, progressively broaden and finally become convex or inverted. These represent the value for a single proportion, e.g., 40/60 (identical and equivalent in magnitude except for sign to 60/40) at progressively larger values of $\Delta M/\sigma$. Thus, a given

TABLE 80. FITTING OF PEAK SEPARATION AS A FUNCTION OF COMPONENT SIZE AND PEAK SEPARATION

n_1	0.100	0.150	0.200	0.250	0.300	0.350	0.400	0.450
$M \sigma$	$\Delta M \sigma$ found							
1.00	1.11	1.13	1.14	1.14	1.14	1.13	1.12	1.12
1.20	1.18	1.19	1.19	1.19	1.25	1.24	1.22	1.21
1.40	1.28	1.33	1.35	1.34	1.52	1.30	1.28	1.26
1.60	1.39	1.47	1.49	1.47	1.52	1.48	1.45	1.43
1.80	1.65	1.74	1.75	1.72	1.67	1.62	1.58	1.56
2.00	1.86	1.95	1.95	1.91	1.96	1.92	1.89	1.88
2.20	2.20	2.27	2.25	2.21	2.17	2.15	2.15	2.16
2.40	2.47	2.52	2.48	2.43	2.54	2.60	2.73	2.86
2.60	2.85	2.85	2.79	2.76	2.81	2.98	3.68	3.64

rations of $\Delta M \sigma$ of 1.0, it is not possible to distinguish differences in component proportions, and, generally, components smaller than 15% are correctly distinguished only in terms of magnitude. A similar interdependence is evident between the value of $\Delta M \sigma$ and n_1 in the measurement of $\Delta M \sigma$. Below values of n_1 equal to 0.150, values for $\Delta M \sigma$ are far more inaccurate than above this proportion. The relationship is most inaccurate at very low or high values for $\Delta M \sigma$.

Choice of the constants used in Equations (1) to (5) was made on the basis of least square fitting of the data points, and it can be seen that this is a compromise between the extremes of composition. Undoubtedly, the inclusion of higher order terms in these

equations would improve the closeness of fit over wider range, but this would make their solution considerably more difficult. Nevertheless, this first approximation makes possible the resolution of two populations to an acceptable degree without assuming that they are of equal size and without a prior knowledge of the distance between their means.

CONCLUSION

Two overlapping populations in a compound distribution can be distinguished and their relative size and the distance between their means may be estimated from the values for the fifth and sixth standard moments of the compound distribution. These relationships apply to any system in which components of the same dispersion and gaussian characteristics are present.

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MEASUREMENT OF ISOTOPE RATIOS DURING COMBINED GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Peter D. Klein, William J. Eisler, and Patricia A. Szczepanik

PURPOSE AND METHODS

There are a number of attractive reasons for using stable isotopes for labeling in place of radioactive isotopes. These include a greater ease of handling, the use of conventional synthetic processes, a greater variety of isotopic possibilities and, most important, their inherent safety and absence of toxicity. Nevertheless, only a small proportion of metabolic and clinical studies make use of stable isotopic labeling. At least part of the reason for such neglect is the lack of measurement techniques with the sensitivity, convenience and speed of liquid scintillation counting, proportional counting, or gamma spectroscopy. Isotope ratio measurements by conventional mass spectroscopy usually require the sample to be in the form of a gas, such as CO_2 or NH_3 , which is bled into the ion source over a period of several minutes; the

ratio is determined from the proportion of ions falling on two fixed collector plates. Such a system requires extensive sample preparation, a far greater quantity of sample than is conventionally used for radioactive measurements, and the adoption of a fixed collector plate configuration.

Many of the problems associated with sample preparation and introduction have been overcome by the use of combined gas chromatography-mass spectroscopy.⁽¹⁾ The gas chromatograph serves to fractionate mixtures into individual components which are introduced sequentially into the ion source of the mass spectrometer. This delivery system permits the use of exceedingly small quantities of material, provided only that they have been made volatile through derivative formation. The problem thus concerns the means of measuring the isotope ratio of a gas chro-

matographic peak during its transit through the ion source.

PROGRESS REPORT

The principle which appears to have the greatest potential for isotope ratio measurements is shown in Figure 164. The mass spectrometer ion beam is adjusted to bring the lighter ion into focus on the detector electron multiplier. This ion may be the molecular ion peak or any other fragment expected to contain the isotopically substituted position. Next, an incremental change in the accelerating field or in the magnetic field is introduced in order to bring the heavier ion of the same type into focus on the detector. This increment is produced by a separate power supply to the accelerating voltage or magnet voltage, and when it is disconnected, the focus of the instrument reverts to the point of focus for the lighter ion. Thus, an alternate addition and withdrawal of this increment will produce alternate sampling of the intensity of heavy and light ions. Such a device has already been described by Sweeley et al.⁽²⁾ for use in the single-focusing LKB gas chromatograph-mass spectrometer, in which the accelerating voltage was used to change focus. The output from the spectrometer was simultaneously switched between two pens of a dual pen recorder to provide a record of the passage of the light and heavy molecular species through the ion source. Because of the slight, but perceptible, degree of isotope fractionation occurring during gas chromatography, the light molecular species emerges from the column ahead of the isotopic molecular species and the true isotope ratio cannot be obtained from a single instantaneous measurement.⁽³⁾ Instead, the individual measurements of ion intensity must be stored separately and integrated to obtain two integral intensities which represent the true isotope ratio.

We have been concerned with the adaptation of the peak-switching concept to the Perkin-Elmer GC-MS 270, a double-focusing instrument in which the electrostatic sector provides beam alignment and focusing and the magnetic field is varied to provide scanning of the mass spectrum. Three questions must be answered to achieve the adaptation: How should the peak switching be carried out? How can the signal be converted to digital values and stored? How can the storage of data be coupled to the peak-switching system? Some of the operational considerations are: the gas chromatographic peak may last as long as 30 sec or be as short as 2 to 3 sec and ideally, each ion peak should be sampled 20 to 30 times during the passage of the gas chromatographic peak. This requires a peak-switching rate of

FIG. 164 Principle of isotope ratio measurements during gas chromatography mass spectrometry.

0.5 to 1.0 Hz variable up to 20 Hz. This, in turn, requires that the instrument respond to a change in focus and stabilize at the new level with settling times of 5 msec or less. Extensive consultation with the design engineers of Perkin-Elmer has indicated that the magnet voltage characteristics are better suited to such rapid switching than the accelerating voltage. The latter circuitry was designed with an emphasis on long-term stability and sacrifices the short-time constants required for switching in favor of drift-free performance; magnetic peak switching modification of the magnet voltage has been carried out and appears to be feasible. We are presently constructing the calibrated voltage supply and the flip-flop circuit required to add and withdraw the incremental magnet voltage.

Control of the switching frequency is achieved by the use of a time base generator which simultaneously switches the voltage and routes the detector signal to an address in a multichannel analyzer. The logic of the address advance has been specially modified to permit sequential storage in alternate halves of the memory. Thus, the address sequence in the modified mode of operation is typically 001, 201, 002, 202, 003, 203, etc. In this manner, the two ion peaks are stored in separate halves of the analyzer and may be read out in individual fractions, or integrated and read out as two sums. The time base generator is also used to provide conversion of the analog signal from the electron multiplier of the detector to a digital form. The voltage from the detector signal is sampled and charges a condenser in the gate cir-

cuitry. Pulses from a 1 MHz oscillator are accumulated in the addressed channel for a time proportional to the charge on the condenser. This system of analog to digital conversion is less accurate than conventional weighing circuits, but has the advantage of being an integral part of the analyzer system. The digitization and storage of test signals has been satisfactorily achieved, as well as subsequent readout in soft copy and punched card format. Coupling of the analyzer to the 026 key punch has been successfully carried out and is operating satisfactorily.

CONCLUSION

It is our immediate objective to complete the remaining linkage between the time base generator and

peak switching circuitry and establish the performance of this concept on known mixtures of deuterated compounds.

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EXAMINATION OF BETA SPECTRA IN A LIQUID SCINTILLATION COUNTER COUPLED TO A MULTICHANNEL ANALYZER

Peter D. Klein, William J. Eisler, and Merlin H. Dipert

PURPOSE AND METHODS

The measurement of beta isotopes in biological material by liquid scintillation counting has one major drawback: the weaker the energy of the isotope, the greater the likelihood that not all of the radiation will be detected. Two factors intervene: The solvent molecules excited by the passage of the beta particle may, instead of transmitting their excitation to the fluor molecules which give rise to photons, give up their energy to a secondary species of molecule. Additionally, before they reach the photomultiplier tube the photons produced may be absorbed and degraded by colored substances in the scintillation fluid. These two processes result in a reduction in the average photon energy and in a distortion of the energy distribution characterizing the isotope. The degree to which a given distribution is displaced or distorted is related to its mean energy and to the predominance of one or the other quenching mechanisms. The conventional means of determining the efficiency with which a sample is being counted is to irradiate the sample with a gamma source ("external standard") held against the sample vial. The gamma radiation produces secondary electrons within the sample, which behave exactly like the beta particles being counted, i.e., they produce excited solvent molecules and photons and are subjected to the same quenching factors as the sample. They are sufficiently energetic, however, to result in photon production under the most adverse conditions and the effect of quench-

ing is almost entirely limited to a reduction in the average energy of the photons. If the proportion of photons with an energy exceeding some mean value is compared to the proportion below this value, the ratio will reflect changes in quenching within the sample. This "external standard ratio" can be plotted against counting efficiency for an isotope under known conditions, and the resulting calibration curve may be used to obtain disintegrations per minute from counts per minute and the external standard ratio. Unfortunately, the beta spectrum of the sample may not respond exactly the same way to all factors reducing the external standard ratio by the same amount, i.e., different calibration curves may be required for the two types of quenching. A clearer understanding of the behavior of beta spectra under various quenching conditions may permit a more explicit formulation of correction factors.

PROGRESS REPORT

The gas chromatograph-mass spectrometer system currently being assembled has provisions for recording spectra in the multichannel analyzer. This analyzer is a 400-channel instrument, coupled through an interface to an IBM typewriter and 026 key punch. The output of phototubes, through the summing amplifiers of the Beckman LS 200 liquid scintillation counter, has been connected to the Packard analyzer through a conventional gating circuit. This combination permits the entire beta spectrum to be stored in

FIG. 165. Beta spectrum of tritiated toluene in the presence (left) and absence (right) of oxygen dissolved in the scintillator solution

the analyzer memory in far greater detail than the two or three channels available in the liquid scintillation counter. This was done long ago in the case of gamma isotopes but characterization of beta spectra as statistical populations seems to have lagged behind the treatment of discrete gamma spectra. (This appears to be a general novelty to other users of liquid scintillation counters. We have found that the display of beta spectra in this manner succeeds in conveying to students an immediate comprehension of the relationship of the tritium spectrum to the carbon spectrum, window selection for dual label counting, effects of quenching, etc.) The advantage of this instrument configuration is that the data may be handled by existing computer routines for statistical analysis and on-line plotting of spectra.

In the short time that this configuration has been in

FIG. 166. Beta spectrum of radiocarbon labeled toluene in the presence (left) and absence (right) of dissolved oxygen in the scintillator solution

operation, no extensive analysis of peak shapes has been possible. A striking qualitative change of known origin is illustrated in Figures 165 and 166, which simply represent the spectra of tritium and radiocarbon in standard scintillation solvents in the presence and absence of dissolved oxygen. The reduction of average photon energy is vivid and graphically evident. Curve-fitting, using normal and log normal distribution functions, has been successful in describing samples with moderate losses in radiocarbon efficiency and extensive losses in tritium. It is further apparent that the present system of liquid scintillation counting, in which sectors of the spectra are examined through a limited number of channels, may be more sensitive to effects generated by a narrow experimental point of view than the multichannel analyzer. Certainly, one can distinguish between loss in efficiency as represented by counts falling within a specific energy range and the failure of those photons to reach the photomultiplier. The second process, as seen in the analyzer, appears to be less severe than the former. This may indicate that *gestalt* has a role to play in the physical as well as the biological sciences.

COMBINED GAS CHROMATOGRAPHY-MASS SPECTROSCOPY OF BACTERIAL FATTY ACIDS

Patricia A. Szczepank, Paul Sacco, William M. O'Leary, and Peter D. Klein

PURPOSE AND METHODS

There is growing interest in the possibility of classifying closely related organisms on the basis of their chemical composition. Such efforts at chemical taxonomy are particularly concerned with distinguishing members of the same species of microorga-

nism, now classified as the same variety, which may have been isolated from different sources. Because the composition of bacteria, particularly the lipid fraction, can be drastically altered by culture conditions of medium and time of harvest, it is necessary to conduct such studies under closely similar

conditions for all varieties. Two laboratories, one in the Department of Pharmacy of Xavier University, New Orleans, and the other in the Department of Microbiology, Cornell Medical College, New York, have been concerned with this classification process as it applies to species of *Pseudomonas*. At Xavier University, there are three varieties of plant pathogens that infect tobacco and at Cornell, there are four cultures of *Pseudomonas aeruginosa*, each isolated from a different source. Drs. Sacco and O'Leary have developed culture conditions for their organisms that are reproducible and comparable within each set. Preliminary characterization of the fatty acid methyl esters has been done in both laboratories, but the absence of definitive standards in several cases has delayed the classification of types of fatty acid. Moreover, the quantitative analysis of composition requires a volume of data handling not available in these laboratories.

PROGRESS REPORT

We have established gas chromatographic and mass spectrometric conditions for the separation and analysis of fatty acid mixtures as their methyl esters, using the Varian Aerograph instrument for quantitative composition and the Perkin Elmer-270 for mass spectra determination. Although it is likely that quantitative analysis of the components can be made from

FIG. 167. Gas chromatogram of the fatty acid methyl esters of *Pseudomonas aeruginosa* #652. Conditions: Column $1\frac{1}{8}$ in $\times$ 6 ft, 10% diethylene glycol succinate on Chromosorb Q, 80-100 mesh; temperature 90 to 185° C 10° per min; gas, helium, 10 ml, min.

FIG. 168 Conditions as in Figure 167. Sample of fatty acid methyl esters treated with trifluoroacetic anhydride.

the total ion current records of the gas chromatograph in the 270, we have not yet established the efficiency with which individual fatty acids are transferred through the molecular separator to the ion source. This has required running all studies on both instruments to obtain both qualitative and quantitative analyses. The previously established gas chromatograph-digital peak integrator system, coupled through a multiplexer to the IBM 026 card punch has permitted calculation of relative retention times and percent composition from the output of the digital peak integrator. This has proved to be an enormous saving of time and the only feasible way of combining replicate runs to obtain averaged values for the composition.

The Perkin Elmer-270, after an extended period of shake down, has produced high quality spectra, which we are classifying and interpreting on the basis of known standards and published spectra. The high degree of reproducibility is shown in Figures 167 and 168, which represent two gas chromatographic records of the same fatty acid mixture; in Figure 167 as the simple methyl esters and in Figure 168 as the trifluoroacetate (TFA) derivatives of the methyl esters. The latter derivative modifies any substituent on the hydrocarbon chain, e.g., an amino or hydroxy group, and reduces its retention time. The letters on the peaks of the chromatograms represent points at which mass spectra were taken, and the listing of

peak indicates the retention time in seconds. When the two chromatograms are compared, the close agreement between retention times is clearly evident and the appearance of three new peaks, resulting from the TFA derivatization, can be ascertained. These are indicated in Figure 168 by prime marks. These chromatograms also illustrate the complexity of the char-

acterization problem: eighteen components can be distinguished in this chromatogram, and each spectrum must be analyzed at least once in each series. Nevertheless, the versatility and potential of this instrument system in the analysis of complex mixtures is clearly evident in the short time it has been operating.

STUDIES ON RAT LIVER RIBONUCLEASES. IV. FURTHER STUDIES ON HETEROGENEITY OF LIVER LYSOSOMES: INTRACELLULAR LOCALIZATION OF ACID RIBONUCLEASES AND ACID PHOSPHATASE IN RATS OF VARIOUS AGES*

Yueh Erh Rahman and Elizabeth A. Cerny

In adult rat liver, by the use of sucrose gradient centrifugation, the intracellular distribution pattern of acid ribonuclease was found to be different from that

of acid phosphatase. This finding further confirms our previous results obtained by zonal centrifugation.

An additional group of subcellular particles in the top few fractions of the gradient was reported in young rats. The biological significance of this new group of particles is discussed.

* Abstract of a paper published in *Biochim. Biophys. Acta* **178**, 61-67 (1969).

STUDIES ON RAT LIVER RIBONUCLEASES. V. LIVER RIBONUCLEASES IN DEVELOPING, 2-ACETYLAMINOFLUORENE FED AND PARTIALLY HEPATECTOMIZED RATS*

Yueh Erh Rahman, Elizabeth A. Cerny, and Carl Peraino

A relationship was found between the activity of three ribonucleases and liver growth.

All three liver ribonucleases showed high activity in fetuses 3 days before birth, in new-borns, and in suckling rats; these enzyme activities decreased gradually and attained the adult level between 30 and 40 days of age. Acid phosphatase was relatively constant during the developing stage of the rat.

Acid ribonuclease (i.e., ribonuclease I) increased soon after feeding 2-acetylaminofluorene while acid phosphatase decreased. Ribonuclease II and ribonu-

lease III increased during the first 5 to 6 weeks and decreased subsequently for approximately 10 weeks before returning to the control level.

In regenerating liver, all three ribonucleases were increased soon after partial hepatectomy with a maximal increase at about 4 hr after the operation. Ribonuclease II activity was significantly decreased from 8 hr to 24 hr. Ribonuclease I and ribonuclease III remained increased up to 72 hr after partial hepatectomy.

The possible implications of the relationship between ribonuclease activities and phenomena of liver growth is discussed.

* Summary of a paper published in *Biochim. Biophys. Acta* **178**, 68-73 (1969).

RNA TURNOVER STUDIES IN LIVERS OF SUCKLING RATS. APPLICATION OF POLYACRYLAMIDE GEL TO RNA SEPARATIONS

Yuch Erh Rahman, Carl Peraino, and Elizabeth A. Cerny

PURPOSE AND METHODS

High activity of liver ribonucleases was found in fetuses three days before birth, in new-borns, and in suckling rats.⁽¹⁾ This finding suggests that the RNA in young rats may be turning over more rapidly than in adult rats. Therefore, experiments were conducted to test the validity of this hypothesis.

Four- to six-day-old suckling rats were injected intraperitoneally with ¹⁴C-*orotic acid* at a dose of 10 μ Ci/100 g body weight. Groups of 16 rats were then killed at different time intervals after the ¹⁴C-*orotic acid* injection; their livers were removed at once and pooled for homogenization in ice cold 0.25 M sucrose. The nuclei were purified according to the method described by Blobel and Potter.⁽²⁾ After the centrifugation, the nuclei were collected from the pellet at the bottom of the tubes; the supernatant, which consisted of the bulk of cytoplasm and cellular membranes, was recovered quantitatively and centrifuged again in the Spinco 30 rotor at 30,000 rpm for 1 hr. This material was used as cytoplasm of the liver. Total RNA was extracted from both the cytoplasm and the nuclei in the presence of sodium dodecyl sulfate, according to

Hiatt,⁽³⁾ and the nucleic acids obtained from both fractions were treated with deoxyribonuclease as described by Di Girolamo et al.⁽⁴⁾ before being precipitated overnight at -20°C from ethanol containing 2% potassium acetate. The precipitated RNA was then dissolved in 0.01 M Tris buffer, pH 7.4.

Aliquots of RNA solution were determined at 260 m μ in a Beckman DU spectrophotometer. A concentration of 10 μ g/ml is assumed to give an absorbance of 0.24 at 260 m μ . The radioactivity was determined in a Beckman liquid scintillation counter.

RNA was further separated by gel electrophoresis, which was performed at pH 9.2 on a 3% polyacrylamide gel (Cyanogum) slab in an EC apparatus. Samples containing 200 μ g of RNA in a final volume between 0.2 ml and 0.6 ml were placed in the sample slots. Migration was toward the anode, and the running time was approximately 2 hr at 300 V and 140 mA. The gel slab was then removed from the apparatus and cut longitudinally into strips, each of which encompassed one sample slot and the migration path of that sample. The strips were then placed in a tray lined with Saran wrap, and the tray was placed on a slab of dry ice to freeze the strips as quickly as possible. Quick freezing was essential to maintain the integrity of the gel structure. Each frozen strip was placed in a specially devised gel slicer (see Figure 169), which was lying on a bed of crushed ice. The frozen strip was then allowed to warm slightly to a subjectively determined optimum consistency for slicing, and slices (1.8 mm) were made as rapidly as possible. Each slice was placed in 2 ml of double distilled water in a test tube, and the tubes were allowed to stand at room temperature for 48 hr. This procedure consistently eluted all the radioactivity from the gel slices and enabled us to obtain highly reproducible radioactivity patterns. The procedure was unsatisfactory with regard to chemical measurements of RNA, however, since variable amounts of extraneous 260 m μ absorbing material was eluted from the gel, which also interfered with the orcinol assay for RNA. It was not possible, therefore, to determine the specific activity of the RNA in each gel slice. The distribution of the RNA in the gel prior to elution was visualized qualitatively in the following manner.

After electrophoresis the gel slab was removed from the electrophoresis chamber and taken to a dark room where it was placed on a Vycor plate (1/8" thick)

FIG. 169. Gel slicer, designed for rapid slicing of semi-frozen gel strips.

FIG. 170 ^{14}C -otic acid incorporation vs. time in total nuclear RNA and total cytoplasmic RNA of rat liver. O, Cytoplasmic RNA; X, nuclear RNA.

which was overlying a sheet of UV-sensitive film. A 256 m μ light source was positioned above the gel and turned on briefly after which the film was removed from beneath the Vycor plate and developed. The position of the UV light source and the time of exposure were varied to produce optimum results.

PROGRESS REPORT

Figure 170 shows the incorporation of ^{14}C -otic acid into the nuclear RNA on one hand and into the cytoplasmic RNA on the other. The nuclei incorporated high radioactivity 30 min after the ^{14}C -otic acid injection, and this incorporation increased rapidly up to 4 hr and then decreased subsequently. Twenty-two hours after the injection, only about one-third of the radioactivity found at 4 hr remained in the nuclei. The cytoplasm, on the other hand, incorporated at a much slower rate, with a maximum after 48 hr, and then decreased steadily up to 140 hr.

Table 81 shows the same results as Figure 170, but they are expressed as percentages calculated from the total radioactivity recovered from the nuclear and the cytoplasmic RNA. Up to 4 hr after the injection of ^{14}C -otic acid, 80 to 90% of the radioactivity was localized in the nuclei, and this radioactivity was then rapidly reduced to a level of 43% 22 hr after the injection; and it was further decreased to 32% after

TABLE 81. INCORPORATION OF ^{14}C -OTIC ACID CYTOPLASMIC RNA AND NUCLEAR RNA

Time	% ^{14}C incorporation in cytoplasmic RNA	% ^{14}C incorporation in nuclear RNA
30 min	7	93
60 min	9	91
90 min	13	87
120 min	16	84
180 min	19	81
240 min	17	83
22 hr	57	43
48 hr	68	32
70 hr	67	33
140 hr	63	37

48 hr. This percentage of radioactivity in the nuclei remained almost unchanged thereafter up to 140 hr.

The above results show very clearly that the most rapidly labeled RNA is mainly localized in the nuclei, and this labeled RNA was subsequently transferred to the cytoplasm. Whether the labeled nuclear RNA was transferred as intact RNA molecules or as degraded materials into the cytoplasm is not known. This point raises the question of whether or not there are RNA degrading enzymes within the nuclei. Experiments are under way in order to clarify this point.

Figure 171 shows the radioactivity found in nuclear RNA and in cytoplasmic RNA at different time

FIG. 171. ^{14}C -otic acid incorporation vs. time in the nuclear *t*RNA and the cytoplasmic *t*RNA of rat liver. O, Cytoplasmic *t*RNA; X, nuclear *t*RNA.

FIG. 172. ^{14}C otic acid incorporation vs. time in the nuclear *r*RNA and the cytoplasmic *r*RNA of rat liver. O, Cytoplasmic *r*RNA; X, nuclear *r*RNA.

intervals after the ^{14}C -otic acid injection. Both showed maximal activity 4 hr after injection, but the nuclear *t*RNA has a radioactivity 4 times higher than that of the cytoplasmic RNA.

Figure 172 shows the radioactivity found in nuclear *r*RNA and in cytoplasmic *r*RNA at different time intervals after the ^{14}C -otic acid injection. The nuclear *r*RNA shows maximal activity 4 hr after injection, but the cytoplasmic *r*RNA shows a continuous increase in its incorporation up to at least 70 hr after the injection.

Figures 173 and 174 are radioactivity patterns in

RNA obtained from two representative gel strips. The electrophoresis procedure was run as described in Method Section. Each figure shows the patterns of a duplicated run. In both figures, a peak activity can be seen between fractions No. 70 and 80, which corresponded to the two low molecular weight RNA bands (see UV absorption patterns Figure 175), but between fractions 20 and 70, radioactivity was found rather uniformly distributed with few small peaks in the middle section of the gels.

The turnover of the total cytoplasmic RNA was found to be around 76 hr, while that of the total nu-

FIG. 173.—A representative radioactivity pattern from the total cytoplasmic RNA after subjection to gel electrophoresis procedure.

FIG. 174.—A representative radioactivity pattern from the total nuclear RNA after subjection to gel electrophoresis procedure.

FIG. 175.—UV absorption pattern of a gel strip after subjection to gel electrophoresis procedure

clear RNA was only 11.5 hr. The fraction of *t*RNA in cytoplasm was however found to have a faster turnover, around 15 hr, whereas both fractions *t*RNA and *m*RNA from the nuclei showed a turnover of 10 and 11 hr respectively; these times are very similar to that of the total RNA.

CONCLUSIONS

The RNA turnover in suckling rats was found to be faster than that of adult rats as reported by other authors.^(5, 6) Loeb et al.⁽⁵⁾ found the turnover of cytoplasmic RNA in adult rat liver to be 5 days, although Revel and Hiatt found the rapidly labeled RNA in cytoplasm to have a turnover time of 40 hr. However, in order to ascertain that our findings with suckling rats are indeed due to the difference in their RNA turnover when compared to that of adult rats and not simply due to difference caused by experimental methods, experiments should also be done with adult rats under our own experimental conditions.

The gel electrophoresis is a more sensitive method for RNA separation when compared to the classical sucrose gradient method. The unsuccessful elution of RNA from the gel for spectrophotometric determinations is, however, a serious drawback.

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PHOSPHOLIPASES A IN PURIFIED SUBCELLULAR FRACTIONS OF RAT LIVER. EVIDENCE OF A MEMBRANE-BOUND PHOSPHOLIPASE IN LYOSOMES*

Yueh Erh Rahman, Jan Verhagen,† Dick T. M. v.d. Wiel,† and Laurant L. M. Van Deenen†

The phospholipases A were investigated in purified mitochondria, lysosomes, plasma membranes, and nuclei of rat liver.

1. Mitochondria showed very low activities of phospholipase A. The enzyme found in this fraction has an optimal activity between pH 6.5 and 7.0, is activated by Ca^{++} , and is specific for the fatty acid at the 2-position (A_2). It cannot be ascertained whether or not the presence of this enzyme in the mitochondria is due to a small contamination by the plasma membranes.

2. Two phospholipases A were found in lysosomes: 1) an acid phospholipase with a pH optimum at 3.5, inhibited by Ca^{++} and Triton X-100, mainly localized in the soluble fraction, and found capable of hydrolyzing the fatty acids at both positions

(A_1 and A_2); 2) an alkaline phospholipase with a pH optimum at 7.5, activated by Ca^{++} , also inhibited by Triton X-100, and found mainly localized in the membranes of the lysosomes. This enzyme was found to be specific for the fatty acid at the 2-position (A_2).

3. Two phospholipases were also found in the plasma membranes: 1) one with a pH optimum at 6.5, specific for the fatty acid at the 2-position (A_2); 2) one with a pH optimum at 8.0, specific for the fatty acid at the 1-position (A_1). Both enzymes required Ca^{++} for their activities. The presence of these two enzymes in the plasma membranes was not due either to contamination by mitochondria or to contamination by microsomes.

4. Purified nuclei were found to have very low phospholipid hydrolyzing activities.

The possible function of the lysosomal membrane phospholipase in the initial binding between lysosomes and membranes of other subcellular particles is discussed.

* Abstract of a paper to be submitted for publication.

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STUDIES ON LIPOSOMES (LIPID SPHERULES) PREPARED FROM MITOCHONDRIAL AND MICROSOMAL PHOSPHOLIPIDS. THEIR SURFACE CHARGE AND THEIR INTERACTIONS WITH VARIOUS PROTEINS

Yuch Erh Rahman

PURPOSE AND METHODS

The important role surface-active properties of lipids play in the transport phenomena of various biological membranes has become well recognized in the recent years. Model systems (e.g., monolayer and bilayer lipid membranes) have been developed and studied. Recently, a method was described for obtaining lipid spherules (or liposomes) from synthetic phospholipids in the presence of water and salt solutions.⁽¹⁾ The structure and sizes of these liposomes were found to be very similar to various membranes of biological particles; they are described as concentric bimolecular lamellae separated from each other by water compartments.⁽²⁾ Diffusions of various ions across these liposomes were also studied.⁽²⁾ The ion transport, as well as lipid-protein interactions, in membranes of subcellular particles is an important phenomenon in cell physiology, and a simple model system, such as the liposomes, obviously seems of interest for such studies; therefore, preparations of liposomes were made from phospholipids of specific subcellular particles, i.e., mitochondria and microsomes, and studies of their surface properties and their interactions with some proteins were conducted.

Mitochondria were isolated from rat liver in a 25 to 45% sucrose gradient; the gradient was centrifuged at $3,500 \times g$ for 2 hr in the International Centrifuge Model PR-6. Purified mitochondria were collected at the level of the 40% sucrose layer. Microsomes were prepared from a 10% rat liver homogenate; the nuclei, mitochondria, lysosomes and microbodies were first removed by a centrifugation at $15,000 \times g$ for 10 min, and the supernatant obtained was then centrifuged at $100,000 \times g$ for 60 minutes. The microsomes at the bottom of the tubes were washed once before they were used for lipid extraction.

Total lipids from purified mitochondria and microsomes were extracted according to Bligh and Dyer;⁽³⁾ phospholipids were separated from the total lipid by silicic acid column chromatography, as described by Sheltawy.⁽⁴⁾

Egg-yolk lecithin was purified by 3 subsequent precipitations as $CdCl_2$ adducts and chromatography on silicic acid. Phosphatidic acid was obtained by enzymic hydrolysis of the egg lecithin with a phospholipase D prepared from extracts of Savoy cabbage. Cardiolipin was obtained commercially.

Liposomes were prepared according to De Gier et al.⁽⁵⁾ Their birefringent patterns were examined under phase microscopy. The electrophoretic mobilities of the liposomes were measured in a cell electrophoretic apparatus. Measurements were made by direct observation of the particle movements in the stationary layer of the cylindrical tube. Mobilities were converted to zeta potentials, using the following equation:

$$\zeta = 12.9 \frac{v}{E},$$

in which v equals velocity and E is the field strength.⁽⁶⁾

Proteins with isoelectric points ranging from acid to alkaline range were chosen; they were bovine serum albumin, ribonuclease, and lysozyme. These proteins were in highly purified forms and obtained commercially.

PROGRESS REPORT

Mitochondrial Liposomes

The formations of mitochondrial liposomes presented no major difficulty; however, addition of 7 mM of $CaCl_2$ facilitated their formation, and they had more uniform sizes when observed under phase microscopy. Zeta potentials of liposomes from 5 separate preparations of phospholipids were as follows: -40.0 , -38.2 , -49.5 , -38.7 , -45.4 mV, with an average of -42.2 mV. Changes of zeta potentials of mitochondrial liposomes in the presence of various proteins are presented in Figure 176.

Microsomal Liposomes

The formations of liposomes by microsomal phospholipids were rapid, and no $CaCl_2$ was needed. Zeta potentials of liposomes from 4 separate preparations of microsomal phospholipids were as follows: -32.4 , -32.1 , -38.9 , -40.2 mV, with an average of -35.9 mV. Their interactions with proteins were similar to those obtained with mitochondrial liposomes.

Liposomes Prepared with Mixtures

Liposomes of comparable zeta potentials to mitochondrial and microsomal liposomes can be obtained by the following mixtures of lipids: a) 85% egg lecithin + 15% cardiolipin, and b) 70% egg lecithin +

FIG. 176. The changes of zeta potentials of mitochondrial liposomes in the presence of BSA, ribonuclease, and lysozyme.

30% phosphatidic acid. In the presence of proteins, changes of zeta potentials of these liposomes were similar to those shown with mitochondrial and microsomal liposomes. In the presence of proteins, zeta potentials of various liposomes prepared by mixtures of lipids showed significant changes only when their starting potentials were higher than -30.0 mV.

CONCLUSIONS

Successful preparations of liposomes from lipids of mitochondria and microsomes were demonstrated.

LIPID ANALYSIS OF PURIFIED SUBCELLULAR FRACTIONS OF RAT LIVER

Yueh Erh Rahman

PURPOSE AND METHODS

Lipids other than those in adipose tissue are located primarily in biological membranes located at the surface of the cells (plasma membranes), and in subcellular particles (mitochondria, microsomes, Golgi apparatus, lysosomes, microbodies, nuclei, etc.).

Analysis of lipid composition of subcellular particles has been reported by many authors (see review paper⁽¹⁾). The values reported in the literature for liver subcellular particles, especially those of the nuclei, are quite variable and contradictory in many cases. The main reasons for this confusion are that a) different methods were used for subcellular fractionations, and a critical evaluation of the purity of these particles was not applied, and b) the methods used to analyze lipid composition often were not en-

This membrane model is a valuable tool to study simple interactions between lipids and proteins. Studies of their interactions with other substances, e.g., carbohydrates or metal ions, can also be extended.

Two interesting facts can be concluded from the experimental results: 1) Lipids from intracellular particles, e.g., mitochondria and microsomes, showed surprisingly high negative charges. 2) A threshold of -30.0 mV in the surface charge of liposomes is needed for measurable ionic interactions with proteins. A physicochemical explanation for this phenomenon is lacking, however. 3) The degree of interactions between the liposomes and protein appears to be determined by the difference of the isoelectric points of the proteins.

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tirely reliable. Therefore, this problem was re-examined using well-characterized subcellular particles, and more recently developed or improved techniques, such as thin-layer chromatography (TLC), column chromatography, and gas-liquid chromatography.

Purified mitochondria, microsomes, and nuclei were used. The preparations of mitochondria and microsomes are the same as those described previously.⁽²⁾ Nuclei were isolated according to the method of Blobel and Potter.⁽³⁾

Total lipid was extracted according to a method described by Bligh and Dyer.⁽⁴⁾ Column chromatography was carried out according to Vorbeck and Marinetti's⁽⁵⁾ method. Fatty acid composition of various lipids were analyzed after esterification by gas-liquid chromatography.

Separation of phospholipids was done with TLC on silica gel G, using a chloroform-methanol-water (65:35:4, v/v) solvent system. Separation of neutral lipids on TLC was done according to the method of Skipiski *et al.*⁽⁶⁾

Total lipid phosphorus was determined by the method of Fiske and Subbarow,⁽⁷⁾ and the cholesterol determination was made with Lieberman and Burckhard reagent.

PROGRESS REPORT

Data obtained for nuclei, mitochondria and microsomes are summarized in Tables 82–85. Phospholipids from nuclei, mitochondria, and microsomes were analyzed by TLC; the results were comparable to those published previously.⁽¹⁾ Electron microscopy of purified nuclei showed that their outer membranes remained intact.

CONCLUSION

Mitochondria and microsomes contained high percentages of phospholipids. This finding is consistent with recently published data.⁽⁸⁾

The fatty acid composition of phospholipids in all three subcellular fractions was similar to various published data.⁽¹⁾ However, the fatty acid composition of the neutral lipids from purified nuclei was not reported by other authors. The interesting features of this composition are 1) high percentages of palmitic

TABLE 82. LIPID ANALYSIS OF NUCLEI, MITOCHONDRIA, AND MICROSOMES OF RAT LIVER^(a)

	Mg lipid/mg protein	Phospholipid, % of total lipid	Cholesterol, % of total lipid
Nuclei	0.019	50.6	2.93
Mitochondria	0.21	88.4	2.80
Microsomes	0.35	78.6	5.30

^(a) Averages from 3 to 7 separate determinations.

TABLE 83. MAIN FATTY ACID COMPOSITION OF PHOSPHOLIPIDS OF RAT LIVER SUBCELLULAR FRACTIONS^(a)

Fatty acid	Nuclei	Mitochondria	Microsomes
16:0	20.23	18.74	13.27
16:1	0.50	1.05	2.78
18:0	25.12	20.23	19.41
18:1	13.11	15.29	12.05
18:2	13.70	20.52	16.58
20:4	21.80	23.15	28.82
22:6	3.20	0.53	5.46

^(a) Area percentages. Averages from 3 separate determinations.

TABLE 84. MAIN FATTY ACID COMPOSITION IN NEUTRAL LIPIDS OF RAT LIVER NUCLEI^(a)

Fatty acid	Area percentage
16:0	30.06
16:1	4.36
18:0	2.24
18:1	21.46
18:2	34.15
20:4	5.15
22:6	1.06

^(a) Area percentages. Averaged from 3 separate determinations.

TABLE 85. LIPID COMPOSITION OF RAT LIVER NUCLEI

Phospholipids: 50.6% (mainly phosphatidyl choline and phosphatidyl ethanolamine)
Neutral lipids: 28.3% (triglycerides: 65%; 1,2 diglycerides: 5–10%; monoglycerides: 25–30%)
Unidentified: 21.1% (phosphorus, hexose, and ninhydrin tests negative)

acid (16:0) and linoleic acid (18:2), and 2) very low percentage of stearic acid (18:0).

The percentage of total phospholipids in nuclei was lower than in most previous reports;⁽¹⁾ this difference can be explained as due entirely to the contamination of the nuclear preparations by other subcellular particles because the conventional differential centrifugation method was the popular choice throughout these studies.

Nuclei isolated under the procedures used in this study conserved their outer membranes, but no conclusion can be drawn concerning the localization of the nuclear lipids.

The cholesterol contents in nuclei were much lower than previous reports,^(9,10) this undoubtedly is due to the contamination by plasma membranes in the preparations. Cholesterol found in mitochondria and microsomes was comparable to Thines-Sempoux's results;⁽¹⁰⁾ in both their and our studies, however, a small contamination in mitochondria as well as microsomes by plasma membranes cannot be ruled out entirely because no ideal marker for the plasma membranes is available. This important point should be clarified in further experiments.

Two interesting conclusions can be drawn from this report.

(1) Neutral lipids in purified nuclei were in greater concentration than in other subcellular particles. The major fatty acids in the neutral lipids are palmitic and linoleic acids.

(2) There are indications that cholesterol might be unique to the plasma membranes.

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CELL GENERATION CYCLE AND RADIATION EFFECTS IN MAMMALIAN CELLS IN CULTURE

Warren K. Sinclair

Techniques with cultured mammalian cells *in vitro* have been widely employed to investigate mammalian cell responses to ionizing and other radiations and agents, as well as to explore regulatory and control mechanisms in mammalian cells. In particular, the use of synchronized populations has permitted these responses to be investigated in relation to the changing biochemistry of the mammalian cell as it progresses through its generation cycle. This program is concerned particularly with relationships between radiation effects and their cell cycle stage and the biochemical factors concerned with these stages.

The following eleven reports embrace the progress made during the past year in studies in mammalian cells with agents which protect or sensitize cells to ionizing radiation, or inhibit particular biochemical processes and the dependence of these upon cell cycle

stage. Similar studies have been conducted with ultraviolet light, and others are in progress upon the interaction between ultraviolet light and X radiation. In addition studies have been carried out on the timing of events in the cell cycle just prior to division. These studies have been confined so far to a single line of Chinese hamster lung cells grown in culture and to some of its derivative sublines. The radiation effects examined include lethal damage, recovery from sublethal damage, division delay, cytological effects, biochemical modifications, and effects upon growth. In most cases populations synchronized by the mitotic selection method, sometimes with the addition of Colemid to improve yield or hydroxyurea to improve synchrony, form the basis of the experimental procedures.

THE CELL CYCLE DISTRIBUTION OF CHINESE HAMSTER CELLS IN STATIONARY PHASE CULTURES

Warren K. Sinclair and Dennis W. Ross

PURPOSE AND METHODS

Investigations of the stationary phase of growth in mammalian cell cultures after the partial depletion of the culture medium have been less complete than those of steady state (exponential) growth. Non-proliferating, stationary phase mammalian cell cultures may represent a closer analog to certain situations *in vivo* (e.g., tumors) than exponential phase cells. The present study was designed to determine the distribution of cells within the metabolic compartments of the cell cycle (M , G_1 , S , G_2) after cell proliferation has apparently ceased in the culture.

The cell cultures were started at a low cell concentration in fresh medium. Cell concentration and average cell volume were monitored during the exponential and stationary phase, using the electronic volume

spectroscopy equipment described previously.⁽¹⁾ Chinese hamster cells of the V79-S171 subline were maintained in two different culture media (HUT-15 and EM-15)⁽²⁾ for this study, because there were indications from cell volume distributions that the cell cycle distribution was likely to differ in the two media. In addition to cell number and average volume, mitotic index, percent of cells synthesizing DNA (by uptake of $^3\text{HTdR}$) and plating efficiency were examined. The distribution of cells within the cycle during early, mid, and late stationary phases was determined by allowing the cells to resume growth in fresh medium containing Colemid as a mitotic blocking agent. A plot of cumulative mitotic index versus time was obtained which, in addition to a plot of cells labeled with $^3\text{HTdR}$ as a function of time, allowed the distribution of the population within the cell

FIG. 177. Growth curve, cell number per culture vs. time, for Chinese hamster cells V79-S171. Open circles for cultures grown in HUT-15 medium, solid circles for EM-15. Divisions of the growth curve into log and stationary phases and the stages of stationary phase are shown above the time axis.

cycle compartments and the length of the cell cycle when growth resumed to be determined.

PROGRESS REPORT

The growth curve, number of cells per culture dish (50 mm diameter) versus time for a typical experiment, is shown in Figure 177. The lengths of log and stationary phases are shown along the time axis. The HUT-15 medium has a composition nearly identical to EM-15, except for an additional enrichment of 2.5% v/v of NCTC 109 vitamin and amino acid mixture⁽³⁾ and 2% v/v of trypsin. The HUT-15 (enriched) medium supports a final concentration of 4×10^6 cells/dish, and EM-15 medium supports 2×10^6 cells/dish. A parameter for describing the amount of cell growth that a particular volume of growth medium can support on these dishes was formulated by the authors. The empirically determined growth curve, cell number versus time, $N(t)$, is integrated from the start (t_0) to the end (t_i) of the log phase of growth. The value of the integral

$$\int_{t_0}^{t_i} N(t) dt = N_{\max}(\text{cell-hours})$$

is the maximum, $N_{\max}$, of cell-hours of steady state growth that the given volume of the medium can support. If $N_{\max}$ is exceeded, the culture ceases log-phase growth and enters stationary phase. For the media used here, these numbers are:

$$\text{HUT-15 } N_{\max} = 8 \times 10^6 \text{ cell-hours/cc}$$

$$\text{EM-15 } N_{\max} = 4 \times 10^6 \text{ cell-hours/cc.}$$

This concept of supportive cell-hours/cc of medium was verified by repeated feeding of dense cultures at increased frequency. If $N_{\max}$ was never exceeded, cell cultures could be maintained in log phase to concentrations of at least 2×10^7 cells/plate.

After the cell medium is depleted to a point where exponential increase in cell number ceases, the cell number remains approximately constant for a period of about 40 hr and then begins to drop slowly. We have divided the stationary phase of cell growth into early, mid, and late stages as indicated on Figure 177. The distribution of cells within the cell cycle was studied in each of these stages.

Figure 178a shows the average cell volume as a function of time for a culture in HUT-15 medium proceeding from log to stationary phase. The average volume drops sharply after inoculation, remains nearly constant during log phase, and then decreases by about 20% to a new low value in stationary phase. Figure

FIG. 178. Average cell volume (relative scale) vs. time for Chinese hamster cells, V79-S171, in HUT-15 medium. (a) Cell volume changes from log to stationary phase; (b) cell volume changes for cells in stationary phase resuming growth in fresh medium.

178b shows the return to log-phase volume within one generation time after cells from a stationary phase population are diluted in fresh medium and resume growth. The volume changes in EM-15 medium followed a somewhat different pattern, possibly indicative of a different distribution of cells in early stationary phase.

The mitotic index of a stationary phase population declined steadily with time after cells left log phase; in EM-15 medium it was about 1% in early, 0.5% in mid, and 0.2% in late stationary phase. Thus, cell proliferation was greatly reduced throughout, but did not cease entirely. Figure 179 shows the results of a growth-resumption experiment from early stationary phase: percent labeled cells and cumulative mitotic index versus time. The cumulative mitotic index curve shows an initial increase after growth resumption, which is interpreted as a burst of cells previously in G_2 now coming into mitosis. A plateau region in the curve follows, lasting 10 to 12 hr, during which time the labeling index (percent of cells in S) rises sharply. The second increase in the cumulative mi-

FIG. 179.—A growth resumption experiment for Chinese hamster cells V79-S171 in stationary phase diluted into fresh medium. Circles and solid line for HUT-15 medium; squares and dotted line for EM-15. Percent labeled cells and cumulative mitotic index vs. time.

FIG. 180. A schematic representation of cell cycle distributions within the metabolic compartments of the cell cycle (M , G_1 , S , G_2) for cells in early and late stationary phase in two media, HUT-15 (enriched) and EM-15 (unenriched). (a) Early stationary phase, HUT-15 medium; (b) late stationary phase, HUT-15 medium; (c) early stationary phase, EM-15 medium; (d) late stationary phase, EM-15 medium.

totic index is presumably due to G_1 cells which, after a very much prolonged G_1 period, reach mitosis. Few cells are stopped in S (DNA synthesis) for cells grown in HUT-15 medium. However, in EM-15 medium, many cells are arrested in S but appear to be nonviable and do not reach the next mitosis when provided with fresh medium (note plateau in Figure 179). This observation was confirmed by the use of cultures treated with hydroxyurea.

The distribution of the cell population in each compartment of the cell cycle, as revealed by these growth resumption experiments is shown schematically in Figure 180 for early and late stages of stationary phase. In HUT-15 medium, the cells are initially arrested in primarily the G_1 and G_2 portions of the cycle. The viability drops rapidly as the time in stationary phase increases. In late stationary phase, all cells are in G_1 but very few are viable. That is, the cells when provided with fresh medium will, after a lag, enter a DNA synthetic period and then proceed through G_2 and form a mitotic figure in a culture blocked with Colcemid. However, these cells will not divide continuously in an unblocked culture and will not give rise to colonies. In EM-15 medium, in addition to cells in G_1 and G_2 , a large fraction is in S . Cells arrested in S are nearly all nonviable and noncycling when provided with fresh medium as discussed earlier. (Note that the distribution of stationary phase cells in the G_1 and G_2 cycle compartments has also

been noted in a mouse lymphoma line.)⁽⁴⁾ Plating efficiency decreases less rapidly in EM-15 medium. There is a steady decline with time in the G_2 and later in the S component in favor of G_1 . In late stationary phase, although only a small portion of the cells were viable, as determined by their inability to form colonies, nearly 50% were able to continue the cycle and reach mitosis when provided with fresh medium. Further studies are needed to identify whether these cells die at mitosis or in some later interphase.

RECOVERY FROM SUBLETHAL DAMAGE INDUCED BY X IRRADIATION IN CYSTEAMINE PROTECTED CHINESE HAMSTER CELLS

Warren K. Sinclair

PURPOSE AND METHODS

Recovery from sublethal damage after X irradiation is now a well-known phenomenon in mammalian cells, provided a shoulder exists in the single dose survival curve.⁽¹⁾ It is also known that thiols, such as cysteamine (MEA), protect some mammalian cells differentially at different stages of the cell cycle,^(2, 3)

FIG. 181.—The response of synchronous Chinese hamster cells irradiated at 6.5 hr (late S phase) with (1800 R + 50 mM MEA) followed by an interval, t , without MEA, then a second (1800 R + 50 mM MEA). The response of cells from 6.5 hr onwards to 1800 R + 50 mM MEA and 3600 R + 50 mM MEA is also shown.

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indicating some form of interaction between the thiol and the biochemistry of the cell. It is, therefore, important to investigate whether thiol-protected cells can also recover from sublethal damage.

It already has been noted that in Chinese hamster cells the shoulder is largest during late S and resistance is maximum there; consequently, recovery is most easily demonstrated in late S phase cells.⁽⁴⁾ Thus, the method is to use synchronous Chinese hamster cells in late S phase, irradiate with a first dose in the presence of MEA, remove the agent and, at various time intervals thereafter, add MEA again, irra-

FIG. 182. The response of synchronous Chinese hamster cells irradiated at 1 hr (G_1 phase) with (1200 R + 50 mM MEA) followed by an interval, t , without MEA, then a second dose of (1200 R + 50 mM MEA). The response of cells from 1 hr onwards to 1200 R + 50 mM MEA and to 2400 R + 50 mM MEA is also shown.

diate with a second dose, then remove MEA. Survival is scored by the subsequent formation of colonies. 250 kVp X radiation, HVL 0.9 mm Cu, at about 160 R/min is employed.

PROGRESS REPORT

The toxicity of two applications of 50 mM MEA was tested in G_1 and S cells and found to be no different from a single application, which usually shows only very slight toxicity.⁽³⁾ Cells in S phase were then irradiated with two doses of 1800 R in the presence of 50 mM MEA with different intervals between the doses. In this interval, no MEA was present. The results are compared with the result of exposure to a single dose of 1800 R in the presence of MEA and a single dose of 3600 R in the presence of MEA (Figure 181). Evidently, marked recovery occurs in the cells irradiated with two doses with an interval between. Thus, recovery from thiol-protected cells in S phase does occur.

A further experiment was conducted to determine

the response in G_1 cells, using smaller doses of 1200 R, 2400 R, and 1200 R + 1200 R respectively. The result is shown in Figure 182. A small amount of recovery may have occurred. However, in both the thiol-protected and the unprotected cell, survival curve shoulders are small and recovery is difficult to prove in G_1 cells. More definitive experiments are needed to establish whether thiol-protected G_1 cells recover as do S cells.

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PROTECTION BY CYSTEAMINE OF CELLS SENSITIZED BY HYDROXYUREA TO X IRRADIATION

Warren K. Sinclair

PURPOSE AND METHODS

Cysteamine (MEA) protects cells most effectively at their most sensitive stage in the cell cycle, i.e., mitosis.^(1,2) Cells blocked at the $G_1 - S$ transition with hydroxyurea (HU) can be rendered as sensitive as cells in mitosis, but this sensitivity develops only over a few hours of blocking with the agent.⁽³⁾ It is of interest to examine whether cysteamine can reverse the sensitizing effects of hydroxyurea when it is added just prior to irradiation.

Synchronous populations of Chinese hamster cells selected at mitosis are employed. Cells are exposed to HU added in G_1 for varying periods, and just prior to 250 kVp X irradiation MEA is added. Both agents are rinsed off immediately after irradiation. In some cases, HU is replaced again in order to examine the post-irradiation effect of HU upon MEA treated cells. Surviving cells are incubated and grow into visible colonies in 8 to 10 days.

PROGRESS REPORT

The results of cysteamine treatment on HU-sensitized G_1 cells are shown in Figure 183. Cysteamine protects these cells [Dose Modifying Factor (DMF)

>5] at least as well as mitotic cells but not quite to the level of un-sensitized cells. Likewise, the post-irradiation effect of HU in MEA-treated cells shown in Figure 184 is reduced, compared with that of untreated cells (shown in Chart 4, Ref. 4). The modified responses indicate that, although cysteamine does not completely erase the effects of HU, there is interaction between the cellular effects of the two agents. This is additional evidence (if it were needed) that MEA is not simply a dose-modifying agent acting via a mechanism such as the suppression of free radicals, but that it interacts directly with cellular constituents, even in the brief time between addition of the agent and irradiation.

In a practical way, it emphasizes the remarkable capacity of cysteamine to protect sensitive cells more than resistant cells and thus tends to level off variations in sensitivity due not only to cell cycle modifications but also due to induction by hydroxyurea.

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FIG. 183. Cysteamine protection of G_1 cells inhibited with hydroxyurea. Curve X, normal age response after 710 Rads. Curve XH, age response of cells treated with 1 mM HU at 1.5 hr and given 710 Rads at times indicated. HU removed immediately after irradiation. Curve XCy, age response of cells treated with 75 mM cysteamine during irradiation with 2360 Rads. Curve XHCy, age response of cells treated with 1 mM HU at 1.5 hr, then with 75 mM cysteamine during irradiation with 2360 Rads.

X-ray damage during the cell cycle of Chinese hamster cells. *Radiat. Res.* **39**, 135-154 (1969).

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PROTECTIVE EFFECTS OF CERTAIN AGENTS AGAINST X IRRADIATION IN MAMMALIAN CELLS

Warren K. Sinclair

PURPOSE AND METHODS

Cysteamine (MEA) protects differentially during the cell cycle.^(1, 2) However, even up to almost toxic levels, MEA does not completely level off the cell cycle response.⁽²⁾ Possibly, a more effective sulfhydryl protective agent could completely level off the response. Alternatively, other types of agents (e.g., disulfide protectors), if they have or do not have a

FIG. 184. Cysteamine protection of G_1 cells inhibited with hydroxyurea showing postirradiation effect of hydroxyurea on cysteamine protected cells. The data are for a different experiment from those of Figure 183, but curves X, XH, XCy, XHCy are for the same conditions and doses as in Figure 183. Curve XHCyH shows the response of cells treated with 1 mM HU at 1.5 hr, then with 75 mM cysteamine during irradiation at ~ 4 hr, then with 1 mM HU present subsequently.

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differential action, may cast light on the differential effect observed for MEA.

The method employs asynchronous cells to establish, first, toxicity and, then, DMF's (dose modifying factor) for the agent before testing for differential action during the cell cycle, using synchronous cells. The agents are added just prior to irradiation and rinsed off after 30 min, and the surviving cells are al-

* FIG. 185. Survival (colony formation) of Chinese hamster V79 cells (asynchronous) after 30-min exposures to various concentrations of radioprotective compounds. (Exp. 338.)

FIG. 186.—Survival (colony formation) of Chinese hamster cells irradiated in the presence of various concentrations of cysteamine.

lowed to grow into colonies during the next 8 to 10 days. 250 kVp X rays, HVL 0.9 mm Cu, are used at about 100 R/min, absorbed dose rate 0.945 Rads/R.

PROGRESS REPORT

The agents investigated so far include MEA, cysteamine (already known to be ineffective in cultured cells),⁽²⁾ AET (aminoethylisothiourea),⁽³⁾ and three compounds (identified as #638, #2529, and #2721) which had shown promise in screening tests in animals at the Walter Reed Army Research Institute.*

The results of toxicity studies on these 6 agents are shown in Figure 185. AET was toxic above ~10

* I am indebted to Dr. David P. Jacobus of the Division of Medicinal Chemistry, Walter Reed Army Institute of Research, for samples of these compounds.

TABLE 86. PROTECTIVE EFFECT AND TOXICITY OF PROTECTIVE AGENTS ON CHINESE HAMSTER CELLS IN CULTURE

Agent	Toxicity	Effect on survival after 750 R Conc. mM Survival	Protection	Approx. DMF
#638 ^(b)	Not toxic up to 50 mM	50 0.14	No effect at 50 mM	~1
#2721 ^(c)	Toxic above 20 mM	20 0.20	Slight protection at 20 mM	~1.2
#2529 ^(d)	Not toxic up to 50 mM	50 0.78	Good protection at 50 mM	~3.8
Cysteamine	Not toxic up to 50 mM	50 0.89	Good protection at 50 mM	~4.5
AET	Toxic above 10 mM	20 0.056	Toxic at 20 mM	
Cystamine ^(e)	Toxic above 5 mM	50 0.066	Toxic at 50 mM	

^(a) Derived from a test after 750 R only and using survival data vs. DMF for cysteamine to estimate approximate dose modifying factor (DMF).

^(b) $\text{H}_2\text{N}-\text{CH}_2-\text{CH}_2-\text{S}-\text{PO}_3\text{H}^- \text{Na}^+$

^(c) $\text{H}_2\text{N}-(\text{CH}_2)_2-\text{NH}-\text{CH}_2-\text{CH}_2-\text{S}-\text{PO}_3\text{H}_2$

^(d)

^(e) $\text{H}_2\text{N}-\text{C}(\text{CH}_3)_2-\text{NH}-\text{CH}_2-\text{CH}_2-\text{SH}-\text{CH}_2-\text{CH}_2-\text{SO}_3^-$

^(f) In earlier experiments, cystamine was found to be only slightly toxic (~0.8) above 5 mM.

mM even when the pH was adjusted (other experiments, not shown here). Thus, it could be used only at comparatively ineffective levels in these cells. It will be noted that in this experiment cystamine was toxic at almost all levels whereas, previously,⁽²⁾ toxicity was not a serious problem. Variability, possibly from batch to batch, is characteristic of some of these agents!

As an index of protective effect against concentration of the agent, results of X-ray experiments for different concentrations of MEA in asynchronous cells are shown in Figure 186.

The approximate DMF's of the other compounds are shown in Table 86. Also, by way of example, the effect of compound 2529, with a DMF of ~3 on synchronous cells is shown in Figure 187. There may be a slight differential effect, similar to that for cysteamine, but much smaller.

It is suspected that unless the DMF in asynchronous cells is high, no differential effect is likely to be observed. Prospects of finding an agent superior to MEA, in this regard, do not appear high. In animals, however, compound 2721, a thiophosphate, has been very effective.⁽⁴⁾ That it is ineffective in culture may

FIG. 187. Effect of compound #2529 on the age response of synchronous Chinese hamster cells, as measured by colony formation. The age response of untreated cells at three dose levels is shown on the left panel. There may be slight evidence of differential protection.

result from the inability of the culture system to liberate thiols from these cells. Thus, the thiol counterpart of the thiophosphate may be a much more useful compound in cell culture.

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SENSITIZATION OF CHINESE HAMSTER CELLS TO X-RAYS BY N-ETHYLMALIMIDE

Warren K. Sinclair

PURPOSE AND METHODS

The observation^(1, 2) that cysteamine protects Chinese hamster cells differentially at different stages of the cell cycle has led to various investigations concerning the role of thiols in modifying radiation damage. It is also known that thiol-blocking agents, such as N-ethyl maleimide or iodoacetic acid, sensitize mammalian cells to X radiation.⁽³⁾ Thus, if these agents sensitize cells differentially during the cell cycle, thiols would be further implicated in the radiation damage process, and additional clues as to the nature of this damage should be derived.

The method employs both asynchronous and synchronous mammalian cells grown on plastic petri dishes in EM15 medium. Treatments with 250 kVp X rays are performed with and without the agents N-ethylmaleimide (NEM) or iodoacetic acid (IAA) during irradiation. The agent is rinsed off immediately after exposure, and the cells are incubated for 8 to 10 days to form visible colonies.

PROGRESS REPORT

Treatments with a range of doses of both NEM and IAA have been performed with asynchronous

FIG. 188. Toxicity of NEM and IAA in Chinese hamster cells exposed to the indicated concentration for 30 min. Survival measured by colony formation.

TABLE 87. RESPONSE OF ASYNCHRONOUS CHINESE HAMSTER CELLS TO X RAYS IN THE PRESENCE OF NEM OR IAA

Curve ^(a)	Agent	Plating efficiency	<i>n</i>	<i>D</i> ₀	Sensitivity factor
I	None	74%	~5	220	1.0
II	0.005 mM NEM	64%	~4	140	~1.6
III	0.01 mM NEM	~6%	~6	110	~2.0
IV	0.006 mM IAA	59%	~4	205	~1.1

(a) See Figure 189.

FIG. 190. Response of synchronous Chinese hamster cells to 710 Rads X radiation in the presence of NEM for 30 min. X, no agent; A, 0.0025 mM NEM; B, 0.005 mM NEM; C, 0.0075 mM NEM.

cells to establish the concentration at which these agents sensitize without undue toxicity. At levels often employed with bacteria (0.1 to 10 mM), both agents were quite toxic after only a 30-min exposure. For sensitization studies, the agent must be relatively nontoxic; thus, the borderline of toxicity for 30-min exposures was established at much lower levels within a relatively narrow range, as shown in Figure 188.

The agents were then used at close to the toxic level, and their sensitization properties were determined in X-irradiated asynchronous cells. Survival data for X-irradiated cells with and without these agents is

FIG. 189. Response of asynchronous Chinese hamster cells to X rays in the presence of NEM or IAA. (See also Table 87.)

shown in Figure 189. NEM had a sensitization factor of 1.60 at 0.005 mM (very slight toxicity), and this factor was not improved greatly at 0.01 mM when only 8% of the cells survived the drug alone. At 0.006 mM, IAA had a sensitization factor of only 1.10. (See Table 87.)

It is proposed to investigate the cell cycle dependence of the sensitization factor for NEM, using synchronous Chinese hamster cells. To date, preliminary experiments have been carried out, and one of these is shown in Figure 190. Evidently, a very dramatic

difference exists in the sensitization properties of NEM in late *S* compared with early *S* cells.

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CYCLOHEXIMIDE- AND RADIATION-INDUCED DIVISION DELAY IN SYNCHRONIZED CHINESE HAMSTER CELLS

Silvia Bacchetti and Warren K. Sinclair

PURPOSE AND METHODS

Ionizing radiation delivered to mammalian cells in culture induces, among other effects, a delay in cell division. This delay occurs in cells irradiated at every stage of the cycle (G_1 , *S*, G_2 , *M*) but its magnitude is dependent upon the cell stage^(1, 2) as well as upon the dose of radiation.⁽²⁻⁴⁾ The division delay is due to two factors, a block of the cells in G_2 and a prolongation of the DNA synthetic period.^(1, 5) The latter results only for cells irradiated in *S*. The universal nature of the G_2 block has prompted more detailed studies of this phase with synchronized cells,⁽⁶⁻¹⁰⁾ and the results obtained have suggested that the block could be due to an inhibition in the synthesis of proteins required for division. In support of this hypothesis are the observations that: 1) G_2 cells have already duplicated their DNA; 2) the mean lifetime of RNA coding for protein essential for division in Chinese hamster cells is about 2.9 hr (i.e., longer than the G_2 phase in this cell line and longer than the delays induced by X-ray doses below 1000 Rads);⁽⁷⁾ 3) ionizing radiation and inhibitors of protein synthesis both induce a block in division, and their last points of action in G_2 coincide;⁽⁶⁻⁹⁾ 4) protein synthesis is required during the period of delay for cells to resume division, while DNA and RNA synthesis are not.⁽⁶⁾ However, the observation that both X rays and protein inhibitors have the same point of action in G_2 does not allow us to conclude that both agents damage the same target in the cell. Similarly, the observation that protein synthesis is required during the delay indicates only that proteins are necessary for the repair of the damage induced by X rays. The purpose

of the experiments presented in this report is to study further the nature of the G_2 block in our cell system by determining the last points of action in G_2 of both X rays and a protein inhibitor (cycloheximide) and by establishing whether the effect of the two agents is synergistic or additive.

In our studies, we used synchronous populations of Chinese hamster cells selected at mitosis after a 2-hr incubation with Colcemid (0.1 μ g/ml). The division delay was determined by counting the cells at hourly intervals during 1 or 2 cycles and by comparing the times of division in the treated populations and in the control. Protein synthesis was determined by pulse labeling the cells with ³H-leucine (22.6 Ci mM; 4 μ Ci ml) for 15 min and by counting, after autoradiography, the number of labeled cells as well as the number of grains per labeled cell. All the other details of the methods have been reported elsewhere.⁽¹¹⁾

PROGRESS REPORT

The effect of the protein inhibitor cycloheximide (CH) on the progression of cells toward division was investigated after addition of three different concentrations of the drug (2, 5, and 50 μ g/ml) at various times in the cell cycle, beginning in mid-*S* phase. In the first series of experiments, the cells were exposed continuously to the drug, and the time and extent of cell division, if any, was determined. We observed that when division occurred in these circumstances it was not delayed, compared to the control population and, also, that the rate of division did not differ from that of the control. However, the number of cells dividing was dependent upon the

time of addition of the drug and upon the concentration used. Addition of CH at any concentration during the *S* phase resulted in no division at all, but addition of the drug in very late *S* and through *G*₂ allowed more and more cells to divide. An example of this for 5 $\mu\text{g}/\text{ml}$ of CH is shown in Figure 191. By relating the number of cells dividing to the time of addition of the drug, we were able to map a last point of action in *G*₂. All the cells prior to this point in the cycle at the time of treatment are inhibited in their progression toward division, but all the cells past this point divide normally. In Figure 192 are summarized the results of these experiments for the three concentrations of CH used, as well as for a dose of X rays (600 Rads). The experiments with ionizing radiation were performed by irradiating the cells at different times in the last part of their cycle, and the dose of 600 Rads was chosen initially as one inducing an appreciable division delay (2 to 3 hr).⁽²⁾ [This dose, however, reduced the survival

FIG. 191.—Effects of 5 $\mu\text{g}/\text{ml}$ of CH on cell division. The times of addition of the drug are indicated by the arrows. ●, control, ○, ▲, △, CH added at 5.5 hr (*S* phase), 8 hr (*G*₂) and 9.5 hr (*G*₂/*M*) after synchronization.

FIG. 192.—Mapping of the last points of action for CH (2, 5, and 50 $\mu\text{g}/\text{ml}$) and X rays (600 Rads) in inducing a block in cell division. The relative times in minutes indicate the time before division (*D*) at which the blocks occur and also the length of mitosis (*M*) and *G*₂.

FIG. 193. Effect of different concentrations of CH on the number of labeled cells and the number of grains per labeled cell during a pulse with ³H-leucine. The drug was added at time zero and the uptake of ³H-leucine was determined after a 15 min pulse. Each experimental point is plotted at the beginning of the pulse. ●, control; ○, 2 $\mu\text{g}/\text{ml}$; ▲, 5 $\mu\text{g}/\text{ml}$; △, 50 $\mu\text{g}/\text{ml}$.

of *G*₂ cells to about 0.1,⁽¹²⁾ while the doses of CH used did not affect cell survival for the duration of the experiments.] The results shown in Figure 192, indeed, indicate a coincidence in the last point of action for 600 Rads and 50 $\mu\text{g}/\text{ml}$ of CH. However, they also showed that different doses of CH have different times of action in *G*₂. This dose-dependent effect is apparently due to the fact the 2, 5, and 50 $\mu\text{g}/\text{ml}$ of CH reduce to a different extent the number of cells synthesizing proteins, although they may affect equally the rate of synthesis (Figure 193). Further experiments will be devoted to investigating the last points of action for several X-ray doses (200 to 1000 Rads) to determine if the similarity observed between 600 Rads and 50 $\mu\text{g}/\text{ml}$ occurs over a wide range of doses.

A comparison between a continuous exposure to CH and an exposure to X rays may not be valid, because X rays are delivered to the cells in a short pulse; therefore, we have performed experiments in which a short pulse of CH was given to *G*₂ cells. Although no division delay was observed at the first posttreatment division, there may have been experimental reasons for this.

Finally, although we have confirmed a coincidence in the last points of action of at least one dose of CH and one dose of X rays, we do not think this observation is enough to establish the hypothesis that both agents act on the same cellular target. In fact, while CH reduces appreciably and rapidly the amount of protein synthesis (Figure 193), no similar effect is found after irradiation. On the contrary, in the latter case a stimulation of protein synthesis at early times after treatment has been observed.⁽¹³⁾ Further

studies are in progress to investigate whether treatment of G_2 cells with CH prior to irradiation results in a synergistic or additive effect on the division delay.

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THE EFFECT OF X RAYS ON THE UPTAKE OF ^3H -LEUCINE IN SYNCHRONIZED CHINESE HAMSTER CELLS

Silvia Bacchetti and Warren K. Sinclair

PURPOSE AND METHODS

In a previous report⁽¹⁾ observations on the changes in DNA, RNA, and protein synthesis after irradiation of synchronized cells were reported. After irradiation of S phase cells with 710 Rads, the uptake of ^3H -leucine continued at its maximal rate during the whole period of division delay (i.e., for longer than the period of retention of cells in S phase), instead of decreasing as in the control population. Because of the relevance of these data to the studies presented in the previous report,⁽²⁾ we have started a series of experiments to investigate the uptake of ^3H -leucine during two postirradiation cycles of cells given 710 Rads in different phases. Some of these experiments are reported here.

The method used to obtain synchronized cells and the labeling procedure have been described previously.⁽²⁾ In the present experiments, the concentration of ^3H -leucine used was either 2 or 4 $\mu\text{Ci}/\text{ml}$ (specific activity 22.6 Ci/mM). Labeling of S phase cells to determine the cell age in the cycle was obtained by pulsing the cells with ^3H TdR (specific activity 14 Ci/mM, 0.16 $\mu\text{Ci}/\text{ml}$) for 15 min.

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Figure 194 shows the results of two experiments in which the cells were irradiated in late S phase. In

the first instance, uptake of ^3H -leucine in both control and irradiated cells was followed for part of the next cycle, and in the second experiment into the third cycle after synchronization. The percent of cells labeled with ^3H TdR identifies the position in the cycle of the population at the time of treatment. The results show that when 710 Rads are delivered in S phase (when the rate of leucine uptake is nearly maximal) the irradiated cells continue to incorporate the labeled precursor at a maximal rate or are stimulated to incorporate it at an even higher rate. This confirms the previous observation.⁽¹⁾ The difference between the two experiments in Figure 194 is probably not due to the amount of leucine employed but could be due to the fact that the cells were irradiated at two different times relative to their cycle of protein synthesis, that is, either immediately before or after they had reached the maximum rate of uptake.

The experiment illustrated in Figure 195 shows the results of 710 Rads delivered when most cells are in late G_2 M (as determined by ^3H TdR labeling and by measurements of cell division). At this time, the uptake of leucine in the control is close to its minimal value and the irradiated cells are, indeed, stimulated in their uptake in the 2 to 3 hr immediately after irradiation. At later times, the control cells in-

FIG. 194. Effects on protein synthesis of 710 Rads delivered in late *S* phase. The exact times of irradiation are indicated in the figures. ●, % of cells labeled with $^3\text{HTdR}$ in the control; ○, number of grains per labeled cell after ^3H -leucine labeling in the control; ▲, number of grains per labeled cell after ^3H -leucine labeling in the irradiated population. Both control and irradiated populations have almost 100% of the cells labeled with ^3H -leucine at all times. In the top figure $2\ \mu\text{Ci}$ ml of ^3H -leucine were used; in the bottom figure the concentration of ^3H -leucine was $4\ \mu\text{Ci}$ ml.

crease their leucine uptake, reaching the high level characteristic of *S* phase; the irradiated cells, however, show this increase 3 to 4 hr later (they also suffer a division delay of the same length).

These preliminary experiments indicate that X rays stimulate the uptake of leucine. Whether this stimulation occurs only in *S* cells, which is a possible explanation of all three experiments, or occurs also in *G*₂ *M* cells remains to be determined. Although the stimulation of leucine uptake presumably means a higher rate of protein synthesis, alteration in amino-acid pool size, due to irradiation, can not be eliminated as a possible explanation.

FIG. 195. Effects on protein synthesis of 710 Rads delivered in *G*₂ *M* phase. ●, % of the control cells labeled with $^3\text{HTdR}$; ○, number of grains per labeled control cell (^3H -leucine, $4\ \mu\text{Ci}$ ml); ▲, number of grains per labeled irradiated cell (^3H -leucine, $4\ \mu\text{Ci}$ ml). 100% of the cells were labeled with ^3H -leucine at all times both in control and irradiated populations.

Stimulation of protein synthesis may be disadvantageous to the irradiated cell, and preventing synthesis with a protein inhibitor (e.g., cycloheximide) may therefore increase cell survival, as has been observed in HeLa cells,⁽³⁾ whereas in Chinese hamster cells survival is only slightly affected.⁽⁴⁾

Further experiments will be devoted to the study of this phenomenon in other phases of the cycle and also to the investigation of the rate of protein synthesis and the survival of cells treated with cycloheximide after irradiation.

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ULTRAVIOLET LIGHT-INDUCED DIVISION DELAY IN SYNCHRONIZED CHINESE HAMSTER CELLS

Antun Han, Warren K. Sinclair, and C. K. Yu

PURPOSE AND METHODS

Previous studies^(1, 2) have established the fluctuations in lethal response of Chinese hamster cells to ultraviolet light (UVL) as they age between one

division and the next. These cells were found to be most sensitive in *S*, less sensitive in *G*₁ and *G*₂, and least sensitive in mitosis. A complete description of the kinetics of cell populations irradiated with

FIG. 196.— Division delay in synchronous Chinese hamster cells irradiated with UVL to levels which result in about 0.6 survival. Open symbols, total cells; closed symbols, survivors. A, Growth of controls. B, Growth of cells irradiated in mitosis (151 ergs. mm²). C, Growth of cells irradiated in *G*₁ (113 ergs/mm²). D, Growth of cells irradiated in mid-*S* (113 ergs/mm²). E, Growth of cells irradiated in *G*₂ (143 ergs/mm²). F, Mitotic index vs. time for cells irradiated in *M*, *G*₁, *S*, and *G*₂ with the same UV doses as above.

TABLE 88. DIVISION DELAY AFTER EXPOSURES TO DOSES OF UV DESIGNED TO REDUCE SURVIVAL TO ~0.6 OR 0.3 SURVIVAL

Time of exposure after synchrony, hr	Stage of the cycle	UV dose, ergs/mm ²	Survival	Time of division after exposure, hr			Division Delay (Δd)		
				Tot. pop.	Clones	Mitotic index	Pop.	Clones	Mitotic index
Control		0	1.0	9.0	9.5	9.7	-	-	-
0	Mitosis	151	0.65	14.0	13.7	14.0	5.0	4.2	4.3
		252	0.30	21.0	19.0	18.5	12.0	9.5	8.8
1.25	G_1	113	0.62	14.8	15.0	16.0	5.8	5.5	6.3
		185	0.34	22.0	16.0	22.4	13.0	6.5	12.7
5.5	S	113	0.60	18.4	19.2	17.0	9.4	9.7	7.3
		160	0.41	23.0	19.0	24.0	14.0	9.5	14.3
8.5	G_2	143	0.62	9.0	9.0	9.0	0	0	0
		198	0.30	9.0	8.5	9.0	0	0	0

UVL requires, in addition to lethality, a knowledge of the age dependent effects of UVL upon division delay. This report is concerned with UVL-induced division delay and its relation to the cell age at the time of irradiation.

The harvesting procedure for synchronized Chinese hamster cells has been described in detail elsewhere.^(1, 2) However, division delay experiments require a large number of cells per sample to ensure a reasonable number of cell counts. Therefore, the method of synchronization was slightly modified; harvesting plates were incubated for 2 hr in Colcemid before synchronization (0.1 μ g/ml) to increase the yield of mitotic cells. This change in synchronization has increased the yield by about 2.5 times. Synchronized Chinese hamster cells, subline V79-S171, were exposed to UVL from a GE germicidal lamp, 8.7 ergs/mm²/sec, at different stages of the cell cycle. Two methods of assaying delay were used.

The first measures division delay by counting the increase in cell number with time. As a function of time after irradiation, single-cell suspensions were made by trypsinization. The contents of two plates were pooled to form one sample, and this was counted electronically to determine the growth of the total population. From this, the suspension was diluted, as needed, and plated into three dishes for colony formation to determine the growth of the viable fraction in the population. The procedures used for the growth of controls were the same, except for the absence of UVL exposure.

The second method determines the average mitotic delay by scoring mitotic index as a function of time after synchronization and by comparing control and irradiated cells. The method has been employed pre-

viously with X rays.⁽³⁾ Exposures were performed with cells attached to the bottom of the dish and with the medium removed. UVL exposures were adjusted to yield approximately the same survival levels at different stages of the cycle, and the induced division delay was examined after exposures which yielded 0.6 or 0.3 survival level.

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Figure 196 shows the length of the division delay after exposures which yielded about a 0.6 survival level. The increase in the number of surviving cells is shown relative to the total number of cells in the population at the time of exposure (normalized to 1.0). The results presented in Figure 196 show that cells irradiated in the middle of S suffered the longest division delay, cells irradiated in G_1 or mitosis have about the same delay in division, and cells exposed in G_2 were not delayed. The results of the second method, in which the mitotic index is scored as a function of time, are shown in Panel F (Figure 196). These results show delays similar to those shown in the other panels.

Growth data presented in Figure 196 indicate that total population and viable cells suffer about the same division delay.

A further experiment was conducted in which the exposures were increased to reduce the survival level to ~0.3 at each stage. The patterns produced are similar to those shown in Figure 196, except that the magnitude of the delays was increased. The results for both 0.6 and 0.3 survival are summarized in Table 88.

At 0.3 survival, the results for mitotic index versus time agree best with those for the total population,

whereas the delay for viable cells is somewhat shorter than these. This suggests that all cells reach mitosis. However, all of the cell increase can be accounted for by the increase in size of the viable cell population; thus, the nonsurviving cells presumably are present during most of the experiment and apparently are stopped at mitosis and do not divide.

Evidently, division and mitotic delay are much larger in magnitude than after X-ray doses that reduce cells to about the same survival,⁽¹⁾ but, as in the case of X rays, division delay is age dependent and the largest delays occur when cells are irradiated

in S. The absence of delay in G_2 is an interesting feature of this study.

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INTERACTION OF X RAYS AND ULTRAVIOLET LIGHT IN MAMMALIAN CELLS

Warren K. Sinclair and Antun Han

PURPOSE AND METHODS

X radiation and UV light cause lethal damage in mammalian cells, and the shape of the survival curves with dose in the two cases is similar. Is the nature of the lesion induced by both agents the same or different, or is there an intermediate degree of interaction between them? Various pieces of evidence

indicate differences as well as similarities between these two types of radiation, a notable difference being in their capacity to repair sublethal damage, well known for X rays⁽¹⁾ and not observed after UV irradiation.⁽²⁾

The question of interaction between these two types of radiation is difficult to explore and interpret if asynchronous cells are used because of the marked difference in the age response for these two radiations.^(3, 4) The method reported here, therefore, consists in using synchronous Chinese hamster cells *in vitro*, selected at mitosis, and irradiating them with combinations of UV and X radiation at known stages of the cell cycle, with only a small interval between them, and then incubating the cells until visible colonies are formed by the survivors. 250 kVp X radiation, HVL 0.9 mm Cu, ~100 R/min, absorbed dose 0.945 Rads/R, and a germicidal UV lamp (G.E.) with mainly 254 nm radiation at 8.7 ergs/mm²/sec were used.

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The age response for X and UV radiation in synchronous Chinese hamster cells is shown in Figure 197.

Doses of X or UV radiation were then selected so that the survival was reduced to ~0.2 with the first dose (X or UV) and by about a further 0.2 with the second dose (UV or X). This required careful selection of doses at all points in the cell cycle. The results of an experiment of this type are shown in Figure 198. Full interaction between the two radiations is taken to mean that if an irradiation with X rays is given at some point on the survival curve

FIG. 197.—The age response of synchronous Chinese hamster cells measured by colony formation after ultraviolet light and after 250 kVp X radiation. The approximate distribution of stages of the cell cycle is shown at the foot of the graph.

below its shoulder, irradiation with UV with various different doses therefore will yield a survival curve that has no shoulder. No interaction is taken to mean that the full shoulder appears in the UV survival curve irrespective of the X radiation dose. Experiments of this type are difficult to reproduce precisely, but the results obtained so far show an intermediate level of interaction at most cell ages. Furthermore, in some experiments more interaction appeared to occur when the UV was given first, rather than X rays. Indeed in the example shown, there may be full interaction between UV and X radiation when the UV is given first in G_1 and G_2 (but not in S) but not when the X rays are given first.

More refined experiments will be necessary to establish whether the degree of interaction is determined by the stage of the cell cycle, whether the order of delivering the exposures affects the result, and the effects of timing between exposures. The resolution of these questions may be very useful in our future understanding of the nature of UV- and X-ray-induced lesions.

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CYTOLOGICAL STUDIES ON CYSTEAMINE PROTECTED CHINESE HAMSTER CELLS IN VITRO

C. K. Yu and Warren K. Sinclair

PURPOSE AND METHODS

A preliminary investigation⁽¹⁾ indicated that 50 mM cysteamine reduced cytological damage due to X radiation at each stage of the cell cycle. The effects examined here include mitotic delay and the number and types of chromosomal aberration (including chromosome and chromatid aberrations). In order to establish the dose-modifying effects of cysteamine quantitatively, and to compare these with survival data, a wide range of exposures is needed.

FIG. 198. —Interaction of UV and X rays given within 3 min of each other, with doses adjusted to give approximately the same survival at each stage of the cell cycle. Top, X rays given first; bottom, UVL given first. In each panel the full lines represent no interaction or full interaction as indicated, estimated for the actual exposures used (see text). The crosses and dashed lines show the experimental points.

Chinese hamster cells were synchronized by selection from a log phase population at mitosis and were irradiated at each stage of the following generation cycle: G_1 , S_E , S_L , G_2 . Cells were prepared at the end of the cycle for cytological examination. Mitotic delay and chromosomal aberrations were investigated.

There were two series of experiments. In one series, cells were irradiated in the presence of 50 mM cysteamine with doses from 125 R to 3000 R. In the other series, cells were irradiated without the pro-

tection of cysteamine at doses between 100 R and 750 R.

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Mitotic delay was reduced when cells were irradiated in the presence of cysteamine, at all stages of the cycle. The calculated dose-modifying factor (DMF) was about 2.5 for all the stages, with the possible exception of G_2 which has a value of 1.9 (Table 89). This value is probably within the experimental error, because synchrony is poorest near the end of the cycle. By way of example, 3 hr of mitotic delay are usually observed in cells irradiated in S with a dose of 500 R. In the presence of cysteamine, 1250 R are required to produce the same amount of mitotic delay.⁽¹⁾

The frequency of chromosomal aberrations was also reduced by cysteamine at all cell cycle stages. The distribution of the total breaks resulting from different types of chromosomal aberrations was very similar to that obtained in the absence of cysteamine, except that the dose required was higher. An example is given in Figure 199. Chromosomal aberrations were separated into those of the chromosome type and those of the chromatid type. Cells were exposed to 500 R without cysteamine (upper panel) or 2635 R with cysteamine (lower panel). The DMF for total breaks was about 5.5 ± 2 for all the stages. However, the degree of resolution achievable between stages is more limited than that obtained in survival studies, and small variations in DMF with cell cycle stage may not be detected.⁽²⁾

Some of the chromosomal aberrations obtained are attributed to 1-hit events, and the others to 2-hit events. The total breaks were therefore analyzed separately to determine the difference in DMF between the two types of aberration.

For 1-hit aberrations a straight line was obtained for frequency vs. exposure, and the DMF was about 8 ± 1 for all stages (Table 89). For 2-hit aber-

FIG. 199.— Frequency of chromosomal aberrations induced by X rays at various stages of the cell cycle after a dose of 500 R (upper panel) and after 2635 R in the presence of 50 mM cysteamine (lower panel).

rations, frequency vs. exposure yields a parabolic curve and the DMF was about 5 ± 2 (Table 89).

Therefore, as far as the limited resolution achieved in this study is concerned, the protection of cysteamine, with regard to mitotic delay and chromosomal aberrations, could be explained by simple dose modification and does not require an explanation based on interactions between cysteamine and cellular constituents. This differs from results obtained in survival studies;⁽²⁾ however the difference could be due only to the limited resolution of the cytological studies. The principal difference between cysteamine protection for increased survival and reduced chromosomal aberrations is in the magnitude of the DMF's. They are clearly different. Furthermore, the DMF for mitotic delay also is different from DMF's obtained when either chromosomal aberrations or survival are the end points being measured.

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TABLE 89. DOSE MODIFYING FACTORS RESULTING FROM THE PRESENCE OF 50 mM CYSTEAMINE DURING IRRADIATION

Cell stage	Dose modifying factor		
	Mitotic delay	One hit aberrations	Two-hit aberrations
G_1	2.5 ± 0.3	9.2 ± 1.5	5.7 ± 2.0
S_k	2.5 ± 0.1	7.4 ± 1.2	5.2 ± 1.8
S_L	2.6 ± 0.1	8.6 ± 1.9	5.1 ± 1.6
G_2	1.9 ± 0.2	6.9 ± 0.8	5.1 ± 1.8

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POLYPLOIDY INDUCED BY X RAYS DURING THE GENERATION CYCLE OF SYNCHRONIZED CHINESE HAMSTER CELLS

C. K. Yu and Warren K. Sinclair

PURPOSE AND METHODS

Induction of polyploid cells by X rays is a well-known phenomenon. Our studies have established that in Chinese hamster cells the frequency of polyploidy induced has the same dose dependence irrespective of the magnitude of ploidy.⁽¹⁾ The mechanism for the formation of polyploidy is believed to be endoreduplication.⁽¹⁾ It also has been reported from this laboratory that only chromosome-type aberrations were found in these endoreduplicated polyploid cells, particularly, in polycentric chromosomes.^(2, 3) They often duplicate themselves and exhibit a "bilaterally identically symmetrical" configuration (BIS chromosomes). This suggests that these polyploid cells are induced by X rays at an early stage of the cell cycle, presumably during G_1 . We have attempted to confirm this by studying the induction of polyploidy at various stages of the cell cycle.

V79-S171 "diploid" Chinese hamster cells were partially synchronized by mitotic selection and then X irradiated with 100 R during the G_1 , S_E , S_L , and G_2 stages of the following cell cycle and observed at various times thereafter. In one set of experiments, cells were labeled with 10 μ Ci/ml 3 HTdR during the irradiation and then collected at various times after synchronization for cytological examination. The materials used for these experiments, the techniques for synchronization and irradiation, and the cytological preparations are the same as have been previously described.⁽⁴⁾

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The results of the experiment described above are shown in Figure 200. Most polyploid cells observed after irradiation were "tetraploid" (4N, twice the near-diploid stemline number) and "octoploid" (8N, quadruple the near-diploid stemline number). A few cells of higher ploidy also were obtained.

After 1000 R to cells nominally in G_1 (37% of the cell population was labeled) about 14% of the cells were polyploid at 60 hr after synchronization. At other times after synchronization, only 1% to 9% of

FIG. 200.—Percent of polyploidy induced by 1000 R X rays in partially synchronized Chinese hamster cells during the cell cycle.

the cells were polyploid. A maximum of about 6% polyploidy was observed for cells irradiated while nominally in S_E . This frequency of ploidy, however, may have resulted from contamination of G_1 cells, because only 73% of the S_E cells were labeled at the time of irradiation. For cells irradiated in S_L (73% labeled) about 2% were polyploid while about 4% were polyploid for cells irradiated in nominal G_2 (41% labeled). It should be noted that in these cytological experiments relatively large numbers of cells are required, and consequently synchrony is only partial compared with techniques used for survival studies. Autoradiographic studies of cells incubated with 3 HTdR indicated that polyploid cells were not labeled. Thus, the polyploid cells nominally observed in S_E , S_L , and G_2 were probably in G_1 when they were irradiated.

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A NUCLEAR MAGNETIC RESONANCE STUDY OF THE STRUCTURE AND INTERACTIONS OF NUCLEIC ACID DERIVATIVES IN SOLUTION. C. ASSIGNMENT AND CONFORMATIONAL PROPERTIES OF RIBOSE HYDROXYL PROTONS

David B. Davies and Steven S. Danyluk

PURPOSE AND METHODS

High-resolution proton magnetic resonance (NMR) studies have yielded a large amount of valuable information about the structure and interaction of base rings in nucleic acid derivatives. For example, extensive chemical shift-pH measurements⁽¹⁾ have established that the base and ribose groups of almost all purine and pyrimidine nucleosides and nucleotides favor an *anti* conformation about the glycosidic (C'-N) bond. NMR measurements have also shown that bases of purine mono- and dinucleotides^(2, 3) undergo both inter- and intramolecular base-stacking interactions in aqueous solution. In dinucleotides base stacking is accompanied by a change in conformation of the ribose groups.⁽⁴⁾

From this work a rather good picture has emerged of the part played by base rings in stabilizing the structure of nucleic acids (DNA, RNA, etc.) in solution. It would be most useful if the data for base rings could be combined with similar results for the ribose phosphate groups. However, such information is lacking at present and, accordingly, as the next step in our NMR study of nucleic acids, a detailed study was undertaken of the conformations and intra- and intermolecular interactions of ribose and deoxyribose groups in a variety of nucleosides and nucleotides. Since the ribose hydroxyl groups undoubtedly exert an important influence upon the conformation of the ribose-phosphate backbone,

the initial part of this study has been concerned with the assignment of signals for these protons. We report on this aspect of the work in the following.

PROGRESS REPORT

The ribose groups of nucleosides contain three ribose hydroxyl protons of interest. These are the O-H_{2'}, O-H_{3'}, and O-H_{5'} protons, Figure 201. In deoxyribose nucleosides the O-H_{2'} group is replaced by a hydrogen.

If proton exchange processes are minimized sufficiently then well-resolved multiplets, arising from spin-coupling interactions with vicinal methine protons, should be observed for hydroxyl protons of both ribose and deoxyribose nucleosides. Although separate hydroxyl signals were observed in an earlier study⁽⁵⁾ the residual H₂O concentration in the dimethyl sulphoxide (DMSO) solvent was sufficiently high to prevent detection of fine structure in the signal.

In the present work the solvent system selected consisted of a mixture of DMSO-d₆ and C₆D₆. Binary mixtures of these solvents were found to give the best resolution of ribose ring and hydroxyl protons. Extreme precaution was taken to eliminate trace amounts of water from the solutions. All of the solvents were dried exhaustively over molecular sieve, and the nucleosides were pumped on a high-vacuum line for extensive periods to remove adsorbed water molecules. Solutions were made up in a dry box and sealed in NMR tubes to prevent absorption of water vapor.

High-resolution spectra were measured at 30°C for 0.1 M solutions of the common purine and pyrimidine nucleosides. Spectra were recorded at 60 MHz with a Varian DA 60I internal-lock spectrometer locked on internal tetramethylsilane.

A typical spectrum for the ribose and hydroxyl protons of adenosine is illustrated in Figure 202. An assignment of the hydroxyl proton signals was carried out in the following manner. Comparison of the spectrum in the dried solvent system with that obtained previously in "wet" DMSO⁽⁵⁾ permitted the assign-

FIG. 201.—Numbering scheme for the ribose and deoxyribose rings of the nucleosides.

FIG. 202. Part of the 60 MHz NMR spectrum of 0.1 *M* adenosine in 40% benzene DMSO solution showing the proton resonance spectrum of the ribose ring and the double and triple resonance spectra used in spectral analysis. The horizontal lines connect coupled signals with the solid arrow indicating the irradiating frequency and the open arrow showing the observing frequency in double and triple resonance experiments.

ment of multiplets at 5.26 ppm, 5.56 ppm, and 5.80 ppm to hydroxyl protons. The remaining signals in the region of the spectrum shown in Figure 202 are due to ribose ring protons.

A further assignment of the signals to individual hydroxyl groups can be made by the use of double and triple resonance techniques. For example, double and triple resonance experiments establish the coupling

scheme $C_1\text{-}H_1$, $C_2\text{-}H_2$, OH_2 ; cf. connected arrows in Figure 202. Hence, an assignment of the doublet at 5.56 ppm to the $O\text{-}H_2$ proton is possible. Similarly an assignment may be made of the signals at 5.80 ppm and 5.26 ppm to the $O\text{-}H_5$ and $O\text{-}H_3$ protons. Assignment of the hydroxyl protons for other nucleosides was made in an analogous manner.

Since the hydroxyl multiplet patterns were all very

TABLE 90. PROTON CHEMICAL SHIFTS IN PPM (± 0.002 ppm) FROM TMS AND SPIN-COUPLING CONSTANTS ($J \pm 0.1$ Hz) OF 0.1 M SOLUTIONS OF THE NUCLEOSIDES IN VARIOUS BENZENE-DMSO MIXTURES AT 30° C

Nucleoside ^(a)	% benzene in solution	Proton chemical shifts, ppm from TMS						Spin-coupling constants		
		H _{2'}	H _{3'}	H _{5'}	OH _{2'}	OH _{3'}	OH _{5'}	H _{2'} -OH _{2'}	H _{3'} -OH _{3'}	H _{5'} -OH _{5'}
T	5	~2.1	4.307	3.603		5.218	5.008		4.3	5.0
dC	5	~2.0	4.208	3.578		5.163	4.931		4.0	5.0
dA	5	~2.5	4.500	3.660	--	~5.3	~5.3	-	4.0	5.0
dG	5	~2.5	4.403	3.603		5.251	4.876		3.8	5.4
U	40	~4.01	~4.01	3.708	5.438	5.117	5.188	4.2	3.5	5.2
C	40	~4.16	~4.16	3.791	5.381	5.003	5.111	4.6	3.5	5.1
A	40	4.213	4.152	3.852	5.563	5.263	5.805	6.0	1.1	5.0
G	20	4.515	4.260	3.695	5.448	5.160	5.101	5.6	4.2	5.0

^a Abbreviations: T, thymidine; dC, deoxycytidine; dA, deoxyadenosine; dG, deoxyguanosine; U, uracil; C, cytidine; A, adenosine; G, guanosine.

close to first order, the chemical shift and coupling constant values were obtained directly from the spectra. A complete summary of chemical shifts and coupling constants is given in Table 90.

Several points may be noted regarding the magnitudes of the coupling constants. A surprising constancy is observed for the H_{5'}-OH_{5'} coupling constants for all the nucleosides. Moreover, the magnitude of this coupling, 5.0 ± 0.1 Hz is close to the values observed for hydroxyl couplings in simple aliphatic alcohols, 5.3 ± 0.2 Hz.⁽⁶⁾ The latter value is very close to the theoretical magnitude expected for a hydroxyl group rotating freely about the C-O bond.⁽⁶⁾ Accordingly, it is reasonable to conclude that the 5' hydroxyl group of nucleosides is rotating freely about the C_{5'}-O_{5'} bond.

In contrast with the 5' hydroxyl couplings, the H_{3'}-OH_{3'} coupling constants vary for different nucleosides, although all of the couplings are significantly less than the freely rotating value. Based on the empirical relationships between J_{HCOH} and the dihedral angle ϕ_{HCOH} ,⁽⁶⁾ and assuming free rotation between *gauche* (2) and *trans* (1) conformers, it can be shown that the O-H_{3'} bond favors a *gauche* conformation relative to the C_{3'}-H_{3'} bond.

The greater variation in coupling constants observed for the H_{2'}-OH_{2'} couplings of the ribose nucleosides reflects the closer proximity of the hydroxyl group to the base ring compared with the OH_{5'} group.

CONCLUSIONS

As part of a continuing study of the proton magnetic resonance spectra of nucleic acid derivatives, measurements were made of the ribose spectra for a number of

nucleosides in ultradry mixtures of DMSO-d₆-C₆D₆. Use of this solvent system has permitted the detection of exchange-free spin-coupled multiplets for ribose hydroxyl protons for the first time, allowing the assignment of hydroxyl signals for all of the common nucleosides.

Based on the magnitudes of the observed coupling constants it is concluded that the O-H_{5'} group is freely rotating about the C_{5'}-O_{5'} bond while the O-H_{3'} and O-H_{2'} groups favor *gauche* conformations relative to the C_{3'}-H_{3'} and C_{2'}-H_{2'} bonds respectively. Finally we note that the assignment of the hydroxyl protons opens up the possibility of a complete analysis of the proton resonance spectra of ribose rings. Work is currently proceeding in this direction.

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THEORETICAL CALCULATIONS OF NUCLEIC ACID CONFORMATIONS. B. CONFORMATIONAL ENERGIES AS A FUNCTION OF ϕ_{C-N} AND $\phi_{C_4-C_5'}$

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PURPOSE AND METHODS

In previous work^(1,2) theoretical conformational energies were calculated as a function of the torsion angle, ϕ_{C-N} , for a variety of nucleosides and nucleotides. These energies were calculated on the assumption that the main contribution to the total energy arises from nonbonded dispersive type interactions. Furthermore, in order to simplify the computational work it was assumed that variables other than ϕ_{C-N} (i.e., 4 for nucleosides; 5 for nucleotides) are fixed. Despite these assumptions, excellent agreement was observed between calculated and observed torsion angles for fifteen nucleosides and nucleotides.

We have now extended the calculations to include the effects of simultaneously changing the values of two variables, ϕ_{C-N} and $\phi_{C_4-C_5'}$, upon the conformational energies of nucleosides. The second variable, $\phi_{C_4-C_5'}$, accounts for rotation about the C_4-C_5' bond of the ribose group. Rotation about this bond may be expected to influence the conformation of the nucleoside primarily by nonbonded interactions between the oxygen atom of the 5'-OH group and the base atoms.

PROGRESS REPORT

As in the earlier work⁽¹⁾ the nonbonded interaction energies were calculated using a Lennard Jones 6-12 potential function,

$$V_{ij} = d_{ij} r_{ij}^{12} - \epsilon_{ij} r_{ij}^6, \quad (1)$$

where V_{ij} is the interaction energy between nonbonded atoms i and j separated by a distance r_{ij} ; d_{ij} and ϵ_{ij} are constants. In a typical calculation, the atomic coordinates obtained from crystallographic data were first converted for all atoms in the molecule into a cylindrical coordinate system with the Z axis directed along the $N-C$ bond. The total conformational energies were then calculated by summing up the interaction energies for all pairs of nonbonded atoms at different ϕ_{C-N} and $\phi_{C_4-C_5'}$ values. In the usual procedure, energies were first calculated at 10° intervals of $\phi_{C_4-C_5'}$, while keeping ϕ_{C-N} fixed. Then energies were calculated at 10° intervals of ϕ_{C-N} with $\phi_{C_4-C_5'}$ fixed. Conformational energy maps were obtained by plotting the energy as a function of the two rotational variables.

An example of a conformational energy map obtained for the pyrimidine nucleoside, cytidine, is given in Figure 203; Figure 204 illustrates the energy map

FIG. 203. Conformational energy map of cytidine. The curves represent points of equal conformational energy in kcal mole⁻¹. Observed ϕ_{C-N} , $\phi_{C_4-C_5'}$ values are denoted by X.

FIG. 204. Conformational energy map of adenosine. The curves represent points of identical conformational energies. The observed ϕ_{C-N} and $\phi_{C_4-C_5'}$ values are denoted by X.

for a purine nucleoside, adenosine. In both figures the calculated conformational energies are plotted as a function of ϕ_{C-N} and $\phi_{C_4-C_5'}$, with the lines denoting connected isoenergetic points. The curves in Figures 203 and 204 may, therefore, be considered as projections of potential energy surfaces on a two-dimensional graph.

From Figure 203 it is apparent that there are a number of well-defined local energy minima in the theoretical energy map of cytidine. However, the four lowest energy minima (-3 kcal mole $^{-1}$) all lie within the ϕ_{C-N} range of -10 to -40° . This is precisely the range corresponding to an *anti* conformation of the base and ribose groups. The theoretical conformation about the ϕ_{C-N} bond, calculated for the case of two variables, is thus very similar to that obtained where only one variable was considered.⁽¹⁾ Rotation of the O-H_{5'} group about the C_{4'}-C_{5'} bond apparently has little influence upon the torsion angle of cytidine.

Although the lowest energy minima are all located within a narrow range of ϕ_{C-N} values, this is not the case with respect to $\phi_{C_4'-C_5'}$. Here, the minima occur in three regions, centered at approximately 60° , 180° , and 300° *. There are, therefore, theoretically at least three possible orientations of the O-H_{5'} group about the C_{4'}-C_{5'} bond. An analysis of molecular model structures and a consideration of the theoretical angles suggests that the three orientations correspond to *gauche*, *trans*, and *gauche* forms, respectively.

The theoretical calculations thus indicate three possible isomers of equal energy for cytidine. In each isomer the base and ribose groups are *anti* to each other; the O-H_{5'} group, however, can exist in either of two *gauche* or one *anti* conformations. It is gratifying to note that the ϕ_{C-N} and $\phi_{C_4'-C_5'}$ values observed crystallographically⁽³⁾ fall in one of the lowest energy minima, i.e., at $\phi_{C-N} = -15^\circ$ and $\phi_{C_4'-C_5'} = 55^\circ$. The crystallographic results are thus in excellent agreement with the theoretical structure.

The conformational energy map for adenosine (Figure 204) is much more complicated than that for cytidine, and shows at least six potential energy min-

ima. Three of the minima correspond to structures in which the base and ribose groups are in *anti* conformations, with the O-H_{5'} group in one of the *gauche* (2) or *trans* (1) conformations. The other three structures correspond to a *syn* conformation, with the O-H_{5'} group again in one of the *gauche* or *trans* orientations.

The energy barriers between the *syn* and *anti* forms are quite small, suggesting the likelihood that the nucleoside can exist as an equilibrium mixture of these forms in solution. A similar result was obtained in previous calculations.^(1, 2) It is interesting to note that the observed ϕ_{C-N} and $\phi_{C_4'-C_5'}$ values⁽⁴⁾ lie within a region of lowest energy, again indicating a satisfactory correspondence between observed and calculated structures.

CONCLUSIONS

Theoretical calculations of nucleoside structures have been extended to include the effects of changing both ϕ_{C-N} and $\phi_{C_4'-C_5'}$ upon the conformational energies. The results for two nucleosides, cytidine and adenosine, have been plotted in the form of conformational energy maps. From these maps it is concluded that the cytidine favors an *anti* conformation with the O-H_{5'} group oriented in one of the *gauche* (2) or *trans* (1) forms. The adenosine, on the other hand, can exist in either the *syn* or *anti* form with the O-H_{5'} group again adopting a *gauche* or *trans* orientation. In both cases it is concluded that rotation of the O-H_{5'} group about the C_{4'}-C_{5'} bond does not significantly alter the orientation of the base and ribose groups about the C-N bond.

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* The values of $\phi_{C_4'-C_5'}$ refer to the vicinal angle between the C_{5'-O_{5'}} and C_{4'-O} bonds.

ELECTRON SPIN RESONANCE STUDIES OF γ -RAY IRRADIATED DINUCLEOTIDES AT 77°K

William A. Bernhard and Steven S. Danyluk

PURPOSE AND METHODS

The physical and chemical changes induced in the primary structure of nucleic acids by ionizing radiation are still not well understood. Previous approaches to the problem have concentrated either on changes in the physical properties of nucleic acid polymers or on the radiation chemistry of individual monomeric nucleic acid constituents. Although both approaches have yielded much valuable information the former suffers from complexities inherent in dealing with large biopolymers while the latter, though yielding definitive results for monomers, may present difficulties in extrapolation to the polymers.

It would be most useful to have data bridging the gap between these two extremes; accordingly, the principal objectives of this study are, 1) to identify free radicals and other species formed upon irradiation of short-chain oligonucleotides (dimers, trimers, etc.) and 2) to correlate these results with monomer and polymer data. The method selected for studying the free radicals is electron spin resonance (ESR), a valuable technique in such studies.

PROGRESS REPORT

Electron spin resonance measurements have been made of the effects of γ -irradiation upon bases,⁽¹⁻⁵⁾ nucleosides,⁽⁶⁻⁹⁾ and nucleotides⁽¹⁰⁾ in the solid state. Irradiation of crystalline samples at various temperatures has permitted the observation of free radicals at different stages in the sequence of events leading from the initial absorption of energy by electrons to chemically stable radiation products. Generally the free radicals observed at low temperatures are replaced with warming by new free radical products assumed to form at later stages in the sequence of events. Furthermore, a correlation is often noted between free radicals produced in the solid state and the radiolysis products in aqueous solution.⁽¹¹⁾

Because the most detailed information is derived from single crystal measurements it was initially hoped to study the effects of γ -radiation upon single crystals of dinucleotides. However, attempts to grow single crystals large enough for ESR work were unsuccessful, and attention was, therefore, directed toward polycrystalline samples.

Commercial samples of the dinucleotides ApA,*

* Nomenclature: ApA = adenyl 3' 5'-adenosine, etc. (cytidine, guanosine, uridine).

ApC, ApG, CpA, and CpU, were obtained from Sigma Chemical Co. and were used without further purification. Each sample, weighing 10 to 20 mg, was dried by evacuation until dry in a quartz tube and then sealed under vacuum. The samples were then γ -irradiated at 77°K at a dose rate of 20 MR/hr for a total dose of 13 MR. Paramagnetic centers produced in the quartz tubes were annealed out of the vacant part of the tube while maintaining the sample part at 77°K. The sample was then transferred to the annealed part of the tube at 77°K, and the tube was placed in a Varian dewar flask containing liquid nitrogen. The dewar flask and sample were inserted in a Varian multipurpose cavity, and ESR spectra were recorded at X-band frequencies with a modified Varian 4500 ESR spectrometer.

An example of a typical spectrum of a γ -irradiated dinucleotide, that for ApC at 77°K, is given in Figure 205(b). The spectrum consists of an intense, relatively broad signal centered near the g -value for diphenyl picryl hydrazyl (DPPH), and a much weaker doublet, $\Delta H = 126 \pm 5$ gauss, located in the wings (arrows), and centered at DPPH. Also shown for comparative purposes is the ESR spectrum for a γ -irradiated polycrystalline sample of 3'-cytidylic acid, Figure 205(a).

The lack of fine structure in the central intense

FIG. 205.—The first derivative ESR spectrum of (a) 3'-cytidylic acid and (b) ApC. Samples were irradiated with gamma rays and observed at 77°K. The magnetic field increases from left to right, the microwave attenuation is 3 db, and the solid lines represent $4 \times$ the gain of the dashed lines.

signal for ApC' and 3'-cytidylic acid prevents a definitive assignment of the signal to a specific type of free radical. The doublets, on the other hand, can be attributed to a free radical which has unpaired spin density on the ^{31}P atom. The existence of such a phosphorus centered free radical was proposed in a previous study on single crystal of 3'-cytidylic acid where Bernhard⁽¹¹⁾ observed a doublet with a nearly isotropic hyperfine splitting of 150 gauss and a g -value varying between 2.004 and 2.054. The unusually large phosphorus hyperfine splitting permits the observation of this radical in polycrystalline 3'-cytidylic acid and ApC'. Additional confirmation of the doublet assignment is given by the observations that the signals are very difficult to power saturate, and decay irreversibly upon warming to room temperature. Both of these properties were also observed for the doublet signal in the 3'-cytidylic acid single crystal study.⁽¹¹⁾

Spectra for the other dinucleotides were similar to those of ApC. However, it was somewhat more difficult to distinguish the outermost doublets in CpU and ApA because of extensive overlap by the central broad peak.

CONCLUSION

A preliminary study has been made of the effects of γ -irradiation upon a series of purine and pyrimidine dinucleotides. From the ESR spectra obtained at 77°K it is concluded that γ -irradiation produces at least two types of free radicals. One of these free radicals can be identified as having the unpaired electron partially localized on a phosphorous atom. This free radical is especially interesting because the phosphorus atom is expected to be a primary ionization site because of its high electron density. Formation of a free radical at the phosphate group of the ribose-phosphate backbone of nucleic acids may thus be

one of the early events in the overall process of radiation damage.

In order to test some of the above ideas further it is essential to identify the structure of the phosphorus-centered free radical. This can best be accomplished by ESR studies of the appropriate single crystals of nucleotides and dinucleotides and work in this direction is currently in progress.

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MOLECULAR INTERACTIONS OF BIOLOGICALLY ACTIVE MOLECULES

William A. Bernhard, Steven S. Danyluk, David B. Davies, and Kunio Hikichi

As outlined in previous reports^(1,2) this study is principally concerned with the determination of precise structures and conformations of biologically important molecules in solution. The ultimate goal is to provide a sound structural basis for the interpretation of inter- and intramolecular interactions of biomolecules.

In order to achieve these objectives extensive spec-

troscopic measurements have been made on a variety of biomolecules including nucleic acids, proteins, peptides, and dyes. Attention has been focused not only upon macromolecules but also upon smaller monomers and oligomers which can serve as model systems for the macromolecules. Various phases of this work have been reported in the literature and in earlier annual reports.

In parallel with the spectroscopic studies we have also been carrying out theoretical calculations of structures and conformations for selected biomolecules. Thus far theoretical structures have been calculated for fourteen mononucleosides and nucleotides, and several dinucleotides. The results of these calculations complement the spectroscopic data in a very gratifying manner.

In the past year we have continued our experimental and theoretical studies of nucleic acid structures. In addition new studies have initiated in the areas of antibiotic structure and interaction, and mechanisms

of radiation damage in nucleic acids. Brief summaries of several aspects of this work are given in following sections.

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HIGH-RESOLUTION MAGNETIC RESONANCE STUDIES OF ANTIBIOTIC STRUCTURES AND INTERACTIONS, PART I. ACTINOMYCIN D: TEMPERATURE AND SOLVENT EFFECTS UPON THE N-H AND NH₂ GROUPS

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PURPOSE AND METHODS

Antibiotics comprise an extremely interesting class of molecules well suited for the study of the relationship between biological structure and function. A great deal of information is now available, not only about the physiological effects but also about the chemical structures of different families of antibiotics.⁽¹⁾ An opportunity is thus afforded for the study of mechanisms of antibiotic action in a wide variety of systems.

Of even greater interest is the possibility of using selected antibiotics as model compounds for studying more complex biological interactions. For example, peptide antibiotics which inhibit messenger RNA synthesis can serve as excellent models for studying the mechanisms of protein repressor action.

There is, however, one drawback to the realization of the full potential of antibiotics as model systems. This is the lack of detailed knowledge about the conformational properties of these molecules. Although X-ray diffraction measurements have been of some use in this direction the structure and conformation in the crystal are not necessarily those most likely to be found in solution. We have, therefore, initiated a broad and comprehensive study of the structures, conformations, and molecular interactions of antibiotic molecules in aqueous and nonaqueous solutions. Among the variety of techniques to be used in this study are high-resolu-

tion nuclear magnetic resonance, electron spin resonance, optical spectroscopy, and analytical ultracentrifugation.

In this initial study our attention has been directed toward the class of antibiotics which acts primarily as RNA polymerase inhibitors. Of these the most important member is actinomycin D, one of more than thirty actinomycins isolated thus far from Actinomycetes microorganisms. Actinomycin D has been the focus of considerable research work⁽²⁾ in the past 25 years, not only because of its tumor-inhibiting ability, but also more recently because of the similarity of its action to that of protein repressors. In this communication we report on some of the results obtained from preliminary high-resolution NMR studies of actinomycin D.

PROGRESS REPORT

In many respects antibiotics are ideal biological molecules for study by high-resolution NMR techniques. The average antibiotic molecule is small enough (M.W. 1000-2000) to minimize line-broadening effects which plague the spectra of proteins and nucleic acids, yet is large enough that one might expect to find some of the regular structural features (secondary and tertiary structures) present in larger biomolecules. If this is the case then complete analysis of the high-resolution proton spectra of moderately large biological molecules may become a reality. This has proved to be the case for actinomycin D (ACD) (Figure 206). In the following we summarize the assignment of the N-H, NH₂ and phenoxazinone ring proton signals of

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Fig. 206.—Structure of actinomycin D in the pentapeptide form. Abbreviations: meval, methyl valine; sar, sarcosine; pro, proline; val, valine; thr, threonine.

ACD and the effects of solvent and temperature upon the signals.

An illustration of the low-field region of the proton single resonance spectrum for ACD in CDCl_3 at 32°C is given in Figure 207A. This region [415 to 495 cycles/sec relative to internal TMS (tetramethylsilane)] is expected to include, N-H, NH_2 , and aromatic ring proton signals. An assignment of these signals can be made on the basis of deuterium exchange, temperature, and decoupling experiments.

Integration of the ACD signal areas in D_2O -free solutions indicates the presence of eight protons in the multiplet pattern illustrated in Figures 207A and 208A. Addition of small amounts of D_2O leads to the disappearance of two sets of doublets labelled a, b and c, d, Figure 208, along with a decrease in signal area of the peak at 454 cycles/sec. The rate of exchange of the doublets differs markedly, however, with the set at higher field (c and d), exchanging much more rapidly than the set at lower field. The exchange rate of both sets is much longer than the rates generally observed for N-H protons in other organic molecules, i.e., amides. After complete exchange the residual signal

Fig. 207. - Proton resonance spectrum of N-H, NH_2 , and ring protons of ACD in CDCl_3 at 32°C . A, single resonance spectrum; B, double resonance spectrum with the second rf field applied at a point 276 and 267 cycles/sec upfield from a and b; C, double resonance spectrum with the second rf field applied at a point 189 and 153 cycles/sec upfield from c and d. Shifts are in cycles/sec to low field from internal TMS.

area, Figure 208D is consistent with the presence of two protons and the AB type pattern, $J_{AB} = 8.0$ cycles/sec and $\delta_{AB} = 5.8$ cycles/sec is clearly due to the phenoxazinone ring protons. The signals susceptible

area, Figure 280D, is consistent with the presence of two protons and the AB type pattern, $J_{AB} = 8.0$ cycles/sec and $\nu_{AB} = 5.8$ cycles/sec, is clearly due to the phenoxazinone ring protons. The signals susceptible to deuterium exchange are attributed to the four N-H and two amino protons.

A more detailed assignment of the N-H signals can be made from a consideration of spin-coupling interactions. Both the threonyl (thr) and valyl (val) N-H protons can couple with single vicinal methine protons, H^α , on the C^α carbon (Figure 206), giving rise to doublets for the N-H signals. However, the H^α_{thr} protons are further coupled to the β methine proton of

the $(CH_3)_2C^\beta$ group, while the H^α_{val} protons are coupled to β methine protons of the $(CH_3)_2C^\beta$ group.

Since H^α_{thr} protons are >1 ppm to low field of H^α_{val} protons,⁽³⁾ it should be possible to assign the N-H protons by appropriate decoupling experiments. The results of such experiments show (Figure 207B, C) that the a and b doublets collapse to single lines when a second rf field is applied at 267 and 276 cycles/sec upfield (i.e., 215 cycles/sec rel. TMS), while the c and d doublets collapse when the second rf field is applied 153 and 189 cycles/sec upfield (275 cycles/sec rel. TMS). Accordingly the a and b doublets, with J equal to 5.7 and 6.1 cycles/sec, are assigned to the $(N-H)_{val}$ protons and the c and d doublets with J equal to 5.8 and 6.9 cycles/sec are due to the $(N-H)_{thr}$ protons. These assignments were confirmed by additional double and triple resonance experiments on the thr and val side chains.⁽³⁾ A further assignment of the N-H protons to individual pentapeptide rings is not possible at present. Although there has been some question as to whether the cyclic peptides are present as a decapeptide ring or two pentapeptide rings, our solvent perturbation studies⁽³⁾ tend to confirm the latter.

Both the $(N-H)_{val}$ and NH_2 signals shift upfield by 8 to 10 cycles/sec with increasing temperature in the range 34° to $70^\circ C$ (in $CDCl_3$), while the $(N-H)_{thr}$ signals, in contrast, shift downfield approximately 4 cycles/sec. Chemical shifts for the other groups showed a negligible temperature dependence nor was there any significant change of the thr and val $HC^\alpha-NH$ and $HC^\alpha-C^\beta H$ coupling constants. An appreciable shift to low field (6 to 40 cycles/sec) was also observed for the $(N-H)_{thr}$ and NH_2 signals in going from $CDCl_3$ to a more polar aliphatic proton acceptor solvent such as CD_3OH . No comparable change was noted for the $(N-H)_{val}$ signals or for signals of other groups. All of

FIG. 208. Effect of D_2O upon the proton resonance spectrum of N-H, NH_2 , and ring protons of ACD in dioxane at $32^\circ C$. A, D_2O -free solution; the N-H doublets are labelled as in Figure 207. B, C, D, solution containing $\sim 5\%$ D_2O after specified time intervals. Shifts are in cycles/sec to low field from internal TMS.

the signals were independent of ACD concentration (0.01 *M* to 0.10 *M*) in the nonaqueous solvents studied.

Several points regarding the ACD structure can be made from the present results.

1. From the magnitudes of the coupling constants it is very likely that the $\text{HC}^{\alpha}\text{-NH}$ protons favor *gauche* and/or *trans* conformations in all four thr and val residues, assuming that the coupling constant dependence upon vicinal angle is of the Karplus type.⁽⁴⁾ Experimental evidence suggesting such a dependence has been reported very recently by Bystrov and co-workers.⁽⁵⁾ A *trans* vicinal relationship is also indicated for the $\text{HC}^{\alpha}\text{-C}^{\beta}\text{H}$ protons of the val and thr side chains. All of these conformations are consistent with the conformations deduced theoretically for a variety of oligopeptides.⁽⁶⁾

2. The absence of any concentration dependence for the ACD signals tends to rule out any significant solute-solute intermolecular hydrogen bonding (N-H groups) or stacking (phenoxazinone rings) interactions in nonaqueous solvents. On the other hand, the temperature dependence of the N-H* and NH_2 signals (in CDCl_3) and the deshielding of the $(\text{N-H})_{\text{thr}}$ and NH_2 signals in a proton acceptor solvent suggest the involvement of these groups in intra- and intermolecular (solute-solvent) hydrogen-bonding interactions. Although a quantitative assessment of the relative contributions for these two types of interactions is not feasible, the presence of N-H splittings and the slow rates of deuterium exchange for these protons favor a rather strong intramolecular interaction. Furthermore, the slower exchange rate and insensitivity to proton acceptor solvents indicates that the $(\text{N-H})_{\text{val}}$ protons are more strongly hydrogen bonded intramolecularly than $(\text{N-H})_{\text{thr}}$ protons. It is interesting to note that Dreiding molecular models of the cyclic peptides show a particularly favorable possibility for intraring hydrogen bonds between $(\text{N-H})_{\text{val}}$ and $(\text{C=O})_{\text{sar}}$.

3. The observation of well-resolved doublets for the N-H signals along with the absence of any significant temperature effect upon the magnitudes of the $\text{HC}^{\alpha}\text{-NH}$ (thr and val) and $\text{HC}^{\alpha}\text{-C}^{\beta}\text{H}$ (thr) coupling constants is indicative of a pronounced conformational rigidity for the cyclic peptide rings in nonaqueous

solvents. This rigidity is further indicated by the relatively broad line-widths† (~ 1 to 2 cycles/sec) observed for the methyl signals. Although this rigidity is due in considerable part to the lactone linkage, an additional stabilizing effect undoubtedly arises from intra and/or interring hydrogen bonds.

CONCLUSIONS

High-resolution proton magnetic resonance spectra have been measured for the chromopeptide antibiotic actinomycin D (M.W. 1255, 86 protons). From results of deuterium exchange and decoupling experiments it has been possible to assign all of the N-H and NH_2 groups and the phenoxazinone ring protons. Based on the magnitudes of the vicinal $\text{HC}^{\alpha}\text{NH}$ coupling constants it is concluded that these protons favor *gauche* conformations in all of the threonyl and valyl residues.

An extensive study was also made of the effect of temperature and solvent upon the N-H and NH_2 chemical shifts. It is concluded that the N-H protons undergo intra- and intermolecular hydrogen bonding interactions, with the former favored by valyl N-H protons and the latter by threonyl N-H's. Molecular models show a particularly favorable possibility for intramolecular hydrogen bonds between the valyl N-H and sarcosyl C=O groups.

The assignment of the N-H protons represents the first step in the complete assignment of the actinomycin D spectrum. In order to achieve the latter objective further deuterium exchange, decoupling, and solvent perturbation measurements are essential. Such measurements are currently in progress.

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* The downfield shifts of the $(\text{N-H})_{\text{thr}}$ signals with increasing temperature appear to be anomalous. However, such a shift change would arise if the $(\text{N-H})_{\text{thr}}$ groups become more deshielded, i.e., lie more in the plane of the aromatic phenoxazinone ring, with increasing temperature.

† The spectrometer resolution under the same operating conditions was better than 0.2 cycles/sec for methyl signals of simple reference compounds.

MUTAGENESIS BY ULTRAVIOLET AND VISIBLE LIGHT IN CONTINUOUS CULTURES

Robert B. Webb and Muekey S. Brown

PURPOSE AND METHODS

Near ultraviolet (330 to 390 nm) and visible (400 to 500 nm) light was shown to induce mutants resistant to bacteriophage T5 in continuous cultures of *Escherichia coli*.⁽¹⁻³⁾ Mutagenesis was proportional to irradiance between 125 and 1000 $\text{erg mm}^{-2} \text{sec}^{-1}$ and independent of growth rate between 0.1 and 0.5 divisions per hour. There was a requirement for oxygen at wavelengths above 350 nm. A preliminary action spectrum showed a broad peak between 350 nm and 500 nm.⁽³⁾

The purpose of this continuing investigation is the elucidation of the mechanisms of mutagenesis by near ultraviolet (UV) and visible light.

Techniques for chemostat cultures used in this report have been described.⁽³⁻⁵⁾ Continuous cultures of *E. coli* B r and *E. coli* B r t (tryptophan-requiring) were grown in a minimal medium (M9) containing an excess of tryptophan (8 mg l when required) and a limiting concentration of glucose (100 mg l). Populations were maintained in balanced growth at concentrations of 1 to 2×10^8 cells ml. Mutation to resistance to the bacteriophage T5 was assayed by plating cells in the presence of excess phage on nutrient agar (Difco) supplemented with sequestered iron. Streptomycin-resistant mutants were assayed by pour-plating in nutrient agar in the presence of 12.5 μg ml streptomycin. All plating was done in quadruplicate. Colony counts were made after incubation at 37°C for 48 hr, except for assay plates for streptomycin resistance, which were incubated for 4 days. Radiant energy sources were 1) low pressure mercury vapor lamp (Penray SC-11) with pin hole shield, 2) Bausch and Lomb High Intensity monochromator with 150-watt xenon arc lamp, 3) 2" $\times$ 2" slide projector with a 200-hr 400-watt bulb (Sawyer 550A) used with a Baird-Atomic B1 interference filter, and 4) visible fluorescent lamps containing two 4-watt Sylvania cool-white bulbs (F4T5 CW).

A Schwarz vacuum thermopile standardized against a National Bureau of Standards lamp was used to measure the irradiance in the near UV and visible ranges. A General Electric germicidal meter calibrated against the Schwarz thermopile was used to measure the low irradiance from the 254-nm source.

PROGRESS REPORT

The induction of resistance to the bacteriophage T5 (T5^r) and resistance to streptomycin (St^r) by far UV

(254 nm), near UV (330 nm) and visible (400 to 750 nm) light in chemostat cultures of *E. coli* B r 1,t is shown in Figures 209-211. At 254 nm and 330 nm, T5^r cells and St^r cells were assayed in the same chemostat cultures (Figures 209 and 210). The two mutation systems were studied in separate parallel cultures for 400 to 750 nm visible light. The ratio of the rate of T5^r induction to the rate of St^r induction was 75 at 254 nm (Figure 209), 160 at 330 nm, and 190 at 400 to 750 nm. These relationships appear to be reproducible, suggesting that the mutagenic processes affecting T5 resistance and streptomycin resistance are different at 254 nm, 330 nm and at 400 to 750 nm.

It is evident from Figures 209-211 that the mean delay of expression⁽⁵⁾ is much shorter for streptomycin resistance than for T5 resistance. The mean delay of expression for St^r appears to be less than one generation, whereas for T5^r it is approximately 2.5 generations at 254 nm, 4.0 generations at 330 nm, and 4.0 generations at 400 to 750 nm.

The possible role of tryptophan in near UV and visible light mutagenesis was investigated through the use of *E. coli* B r, a strain that does not require tryptophan or any other amino acid. Figure 212 shows the

Fig 209. The induction of T5 resistant cells and streptomycin resistant cells by 254 nm UV in a chemostat culture of *E. coli* B r 1,t. Growth rate was 0.31 divisions per hour.

FIG. 210.— The induction of T5 resistant cells and streptomycin resistant cells by 330 nm UV in a chemostat culture of *E. coli* B/r, 1, t. (Growth rate was 0.28 divisions per hour.)

FIG. 211. The induction of T5 resistant cells and streptomycin resistant cells by 400 to 750 nm visible light in two chemostat cultures of *E. coli* B/r, 1, t. (Growth rate was 0.29 divisions per hour.)

response of this strain to near UV (340 nm) and visible (420 nm) light. *E. coli* B/r shows a much higher mutagenicity than *E. coli* B/r, 1, t or *E. coli* WP2 her⁻ to both near UV and visible light. It is evident that exogenous tryptophan is not the chromophore for near UV or visible light mutagenesis.

Blue light (420 nm) is somewhat more mutagenic

FIG. 212. The induction of T5 resistance by 340 nm UV and 420 nm visible light in chemostat cultures of *E. coli* B/r. The growth rates were 0.21 and 0.23 divisions per hour.

than near UV (340 nm) for *E. coli* B/r. This result is consistent with the preliminary action spectrum reported for *E. coli* B/r, 1, t.⁽³⁾

CONCLUSIONS

Near UV and visible light are mutagenic for both T5 resistance and streptomycin resistance in chemostat and Nephelostat⁽⁶⁾ cultures of *E. coli*. Mutation to T5 resistance is induced at 75 times that of streptomycin resistance by far UV (254 nm). This ratio is somewhat higher for 330 nm UV (160) and 400 to 750 nm visible light (190). The basis of these differences is under investigation.

Added tryptophan is not required for near UV visible light mutagenesis (Figure 212). Furthermore, the much higher mutation rate to T5 resistance shown by *E. coli* B/r, a strain that does not require tryptophan or any other amino acid, shows that neither near UV nor visible light mutagenesis involves a defect in the metabolic pathway of tryptophan.

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GROWTH AND MUTAGENESIS IN THE NEPHELOSTAT

Robert B. Webb and William J. Eisler, Jr.

PURPOSE AND METHODS

A photocell-controlled continuous culture instrument (Nephelostat) developed at Argonne^(1,2) maintains a population density to within $\pm 2\%$ in unrestricted growth, provides a continuous record of growth rate over small increments of time, has a self-contained electronic console with front panel controls, and a detector mechanism that provides a convenient reproducible initial setup without optical focus problems. In the Nephelostat, particle density (scattered light) is monitored and turbidity of the culture is held constant by controlling the rate that fresh nutrient medium is added to the culture. The culture volume is held constant by an overflow siphon.

The Nephelostat has been used in a number of investigations at Argonne, including circadian rhythm studies in *Tetrahymena pyriformis*,⁽³⁾ physiological studies in HeLa cells,⁽⁴⁾ and morphological studies of *Vocardia corallina*.⁽⁵⁾ These applications have shown that with only minor modification anticipated in the design of the instrument, a wide variety of cells can be maintained in balanced growth.

In this investigation, growth and mutagenesis were studied in Nephelostat cultures of bacteria as an extension of parallel studies in chemostat cultures.⁽⁶⁻⁷⁾ Biological techniques in Nephelostat and chemostat experiments are similar. Chemostats are the Kubitschek modification⁽⁸⁻⁹⁾ of the Novick and Szilard design.⁽¹⁰⁾ Growth vessels for both Nephelostat and chemostat techniques are 23 to 24 ml with an inside diameter of 15 mm, with a capillary tube affixed to the bottom to provide aeration and stirring by bubbling air through the suspension. Both Nephelostats and chemostats are operated in individual light-tight incubators with the temperature of the growth vessel controlled to $37 \pm 0.1^\circ\text{C}$. Some of these continuous cultures utilize a refrigerated sample collector, also developed at Argonne.⁽¹¹⁾

Escherichia coli strains B r, B/r 1.t, WP2, and WP2 her⁻ were used in the continuous-culture experiments. The growth medium was M9 salts with glucose

(100 mg/l for chemostats and 100 mg/l, 250 mg/l, or 5 g/l for Nephelostats) and required added amino acids. Ultraviolet light (UV) at 254 nm was supplied by a Penray lamp (Se 11) with a pin hole shield. Irradiance was measured with a General Electric Germicidal Meter calibrated against a Schwarz vacuum thermopile, which had been calibrated against a National Bureau of Standards lamp for the 254-nm wavelength.

PROGRESS REPORT

Development of the Nephelostat

The basic instrument has been improved by 1) the addition of a voltage regulator to the exciter lamp circuit (making it less sensitive to variations in the line voltage), 2) replacement of mechanical meter relay with an electronic type of greater reliability, 3) the addition of an analog recorder that records the density of cells in suspension, and 4) the additional provision of an interchangeable cycle timer.

Specific modifications have been made in the basic Nephelostat for special applications by different investigators. A completely jacketed housing for the growth vessel, nutrient inlet and aeration tube, was designed to hold temperature within $\pm 0.05^\circ\text{C}$ and to eliminate the need for an incubator.⁽¹²⁾ A shutter was added to the housing so that the culture could be illuminated for specific times during the 24-hr cycle.⁽¹²⁾ The simple gravity nutrient feed system and overflow siphon of the basic instrument was replaced with a peristaltic pump for tissue culture studies. In this application the culture volume was increased to 500 ml. The basic detector and control system of the Nephelostat were designed to allow for this alternate nutrient feed method and the increased culture volume.⁽¹³⁾

Growth studies in the Nephelostat

The growth record of a Nephelostat culture of *E. coli* B r 1.t at several cell concentrations is shown in Figure 213. Cultures of *E. coli* can be maintained in balanced growth from 5×10^5 cells/ml to approxi-

FIG. 213. The growth record of a Nephelostat culture of *E. coli* B/r 1, t on an M9 salts-glucose (2 g/l) medium. Growth vessel was 24.5 ml and sample volume was 0.58 ml. One cell generation is registered by 29 events.

mately 6×10^8 cells/ml. The lower limit of the Nephelostat, below 5×10^5 cells/ml, depends on the absence of noncellular reflective particles from the medium. The upper limit of the control system is well above the density so far achieved with any cell system in the instrument. Although batch cultures of *E. coli* on a glucose-salts medium (e.g., M9) can reach 2×10^9 cells/ml, the maximum concentration at which this strain of *E. coli* can be maintained in balanced growth is approximately 7×10^8 cells/ml. Above 7×10^8 cells/ml, the growth rate declined slowly over 2 or 3 generations, after which growth stopped completely. When the culture was diluted below 4×10^8 cells/ml, the growth rate returned to maximum (1.3 generations/hr) after a lag of 2 to 5 hr. The growth rate was found to be constant with *E. coli* B/r 1, t between 5×10^5 cells/ml and 3.5×10^8 cells/ml.

A provisional explanation of these effects is the production of a growth inhibitory substance which, at sufficiently high levels, induces a stationary growth phase in bacteria. In continuous cultures, this substance can approach, and exceed, the concentration required to induce a stationary growth phase at much

lower cell concentrations than in batch cultures. That stationary growth phase is not induced by exhaustion of a required nutrient is shown by the following results.

When an amount of glucose was present that was limiting at approximately 2×10^8 cells/ml (100 mg/l), the maximum growth rate of 1.30 generations/hr could be maintained at cell concentrations of 90%, or less, of the maximum concentration. When the culture was "released" and allowed to reach maximum concentration with subsequent cessation of growth, a dilution of 10% or more, after as much as 24 hr, resulted in the immediate resumption of growth with the maximum growth rate being established within less than 10 min.

The cessation of aeration of the culture resulted in the cessation of growth within 20 min, presumably from the shortage of carbon dioxide which is an absolute requirement of *E. coli*. Resumption of aeration after as long as 24 hr resulted in the immediate resumption of growth, with the maximum growth rate established in less than 10 min with no evidence of a lag. Therefore, the exhaustion of glucose, oxygen or carbon dioxide does not of itself induce stationary phase in *E. coli*.

The effect of continuous irradiation with 254 nm UV on the growth rate of Nephelostat cultures of *E. coli* WP2 in a quartz growth vessel is presented in Table 91. There is only a very small decline in growth rate in a continuous irradiance of $0.036 \text{ erg mm}^{-2} \text{ sec}^{-1}$. Although the irradiance is small, this intensity results in an accumulated incident dose of 145 erg mm^{-2} generation. An irradiance of $0.15 \text{ erg mm}^{-2} \text{ sec}^{-1}$ resulted in a growth rate reduction of about 15%. The culture appeared to be able to grow indefinitely in this irradiance of 254 nm UV. This irradiance gives an accumulated incident dose of 470 erg mm^{-2} generation. A dose of 470 erg mm^{-2} will inactivate 80% of a stationary phase culture of this strain. As not more than 10% of the population was incapable of colony production, even after many hours of exposure, damage induced by low levels of 254-nm UV in *E. coli* in free growth is not cumulative. It is evident that *E.*

TABLE 91. GROWTH OF *E. coli* WP2 UNDER CONTINUOUS IRRADIATION WITH 254 NM UV

Irradiance, $\text{erg mm}^{-2} \text{ sec}^{-1}$	Growth rate, divisions hr^{-1}
0	1.30
0.036	1.25
0.15	1.15
0.35	0*

* Growth stops after 3 or 4 mass doublings.

coli in balanced free growth can efficiently repair damage induced by 254-nm UV at irradiances below $0.15 \text{ erg mm}^{-2} \text{ sec}^{-1}$.

Mutagenesis in the Nephelostat

Spontaneous mutation to resistance to the bacteriophage T5 in *E. coli* B/r 1,t in the chemostat (0.20 generations/hr) and in the Nephelostat (1.30 generations/hr) is shown in Figure 214. Although the mutation rate *per hour* is greater in the Nephelostat, it is somewhat less *per generation*. Kubitschek and Bendigkeit⁽¹³⁾ have shown that mutation to T5 resistance (also T6 resistance) under carbon limitation was proportional to growth rate. The results with the Nephelostat are generally consistent with the chemostat results.

Caffeine induction of T5 resistance in the Nephelostat extends the results previously reported for the chemostat (Figure 215). Although the mutation rate per hour was much higher (30 mutants per 10^8 bacteria per hour) in the Nephelostat than in the chemostat (8 mutants per 10^8 bacteria per hour), the mutation rate *per generation* was approximately the same in the two systems. Figure 216 is a plot of chemostat and Nephelostat data for the caffeine induction of T5 resistance over a wide range of growth rates. It can be seen that the relationship obtained by Kubitschek and Bendigkeit for carbon-limited chemostats⁽¹³⁾ extends to the free growth characteristic of the Nephelostat.

These results are consistent with the caffeine induced mutational event occurring as a mistake during replication of DNA. Although caffeine has been shown not to be incorporated in DNA, its similarity to ade-

FIG. 214.—Accumulation of spontaneous phage T5 resistant mutants in Nephelostat and chemostat cultures of *E. coli* B/r 1,t on M9 salts-glucose medium. The chemostat medium contained 100 mg/l glucose, and the Nephelostat contained 250 mg/l glucose.

FIG. 215.—The induction of phage T5 resistant mutants by caffeine in a Nephelostat and chemostat culture of *E. coli* B/r 1,t. The chemostat medium contained 100 mg/l glucose, and the Nephelostat medium contained 200 mg/l glucose.

FIG. 216.—The relationship between the caffeine-induced (450 mg/l) mutation rate and generation rate in chemostat and Nephelostat cultures of *E. coli* B/r 1,t. The line is taken from data of Kubitschek and Bendigkeit.⁽¹⁾

nine and guanine suggests the possibility that caffeine interferes with precise base pairing during DNA replication.

The induction of phage T5 resistance, streptomycin resistance, and tryptophan independence by 254-nm

FIG. 217.—The induction of T5 resistance, streptomycin resistance, and tryptophan independence in a single Nephelostat culture of *E. coli* WP2 by 254-nm UV. Growth rate was 1.30 divisions per hour.

FIG. 218.—The induction of T5 resistance and streptomycin resistance by visible light (460 ± 10 nm) in a Nephelostat culture of *E. coli* B/r1, try⁻. Growth rate was 1.25 generations per hour.

UV in *E. coli* WP2 in the same Nephelostat culture is shown in Figure 217. The 254-nm UV mutation rates for T5 resistance and streptomycin resistance are somewhat higher under free growth in the Nephelostat than under restricted growth in the chemostat. In the chemostat, the induction of T5 resistance by 254-nm UV is independent of growth rate.⁽¹³⁾ Reversion to tryptophan independence has not been studied successfully under chemostat conditions, as revertants have not been observed to accumulate in chemostat cultures. Therefore, the Nephelostat extends the range of mutants that can be studied in continuous cultures to the class typified by the reversion from tryptophan dependence to tryptophan independence.

The induction of T5 resistance and streptomycin resistance by visible light (460 ± 10 nm) in the Nephelostat is shown in Figure 218. This result demonstrates that visible light mutagenesis is not limited to restricted growth in the chemostat. T5 resistance is induced at approximately 150 times the rate for streptomycin resistance. This is the same proportionality observed in chemostat cultures with visible light. Also, the relationship between the two mutation rates with visible light is similar to that obtained with 254-nm UV (Figure 217).

CONCLUSIONS

The Nephelostat has been employed successfully in a variety of biological systems by several investigators at Argonne. It is to be expected that any organism that can be grown in liquid medium, exists as isolated cells (small uniform clumps or short filaments), and that can be prevented from adhering to surfaces of the growth vessel, can be grown successfully in the Nephelostat.

Mutation studies, including spontaneous, caffeine, far UV and visible light induction, in the Nephelostat have extended previous chemostat studies to much faster growth rates characteristic of the Nephelostat. Spontaneous mutagenesis and caffeine induction of T5 resistance in *E. coli* grown in a glucose-minimal salts medium remain approximately proportional to rate of cell division from very slow chemostats to 1.3 generations per hour in the Nephelostat. Far UV mutagenesis to T5 resistance, which is independent of growth rate in the chemostat, increased by a factor of two under free growth in the Nephelostat; however, this increase is much less than that required to give a mutation rate proportional to growth rate. These observations are under investigation.

The Nephelostat affords the opportunity to study three classes of mutation in the same continuous culture. This approach will be exploited in the study of

the nature of mutation induction by far UV, near UV, visible light, and various chemical mutagens.

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EFFECTS OF ULTRAVIOLET AND VISIBLE LIGHT ON A MULTIPLE REPAIR-DEFICIENT STRAIN OF *ESCHERICHIA COLI*

Mickey S. Brown and Robert B. Webb

PURPOSE AND METHODS

The sensitivity of bacteria to ultraviolet light (UV) can be greatly enhanced by altering certain genetic markers of the cells.⁽¹⁻³⁾ In this study, the relative sensitivities of several strains of *E. coli* to radiant energy of different wavelengths were compared. The comparison was made on the surviving fraction of cells after varying exposure times to far UV (254 nm), near UV (365 nm), and visible light (460 nm). This study is an extension of work on effects of near UV and visible light previously reported.^(4,5)

Three strains of *Escherichia coli* were used in this study: *E. coli* K12 AB2480 (has a genetic marker missing for recombination repair) *E. coli* WP2, and *E. coli* WP2 her⁻. *E. coli* K12 AB2480 is of special interest because of its extreme sensitivity to 254-nm UV: the D_{37} of only 0.2 erg mm⁻² for this strain will produce an average of 1.3 pyrimidine dimers in the DNA of a cell of *E. coli*.⁽³⁾ For each series of experiments, the cultures used originated from a single colony isolate. For individual experiments, a suspension of cells was made in M9 salts buffer by removing a small loopfull of bacteria from a 48-hr (37°C) nutrient agar (Difco) slant and aseptically adding the cells to the solution with vigorous agitation. The final concentration after a 1:10 dilution was approximately

10⁷ cells ml. Assays were carried out by making appropriate dilutions from the irradiated samples and plating 0.2 ml of the sample onto nutrient agar plates. Plating was primarily in triplicate with an incubation period of 48 hr at 37°C before colony counts were made.

Dose-response curves were made using three different energy sources. For the far UV (254 nm), a low pressure mercury lamp (Penray SC 11) with an added filter to reduce the long wavelength UV and visible light to very low levels was used. The near UV source was a Bausch and Lomb high intensity monochromator with a 200-watt super pressure mercury arc lamp. The visible light source was a General Electric Mare 300 "metal" vapor arc lamp, (300-watt) with an integral reflector.⁽⁶⁾ This very high intensity source was used with a condensing lens system, a 460-nm peak interference filter (Baird Atomic B 3 with a 20-nm bandwidth at half-peak transmission), and a 750-nm infrared filter. Photoreactivation was carried out with a Sawyer slide projector utilizing a 500 W quartz iodine lamp and an additional lens to reduce the field size. An interference filter (Baird Atomic B 3) was used to isolate the spectral region between 415 and 445 nm.

Irradiance measurements in the near UV and visible

ranges were made with a YSI-Kettering radiometer. The far UV was measured with a General Electric germicidal meter. Both energy-measuring devices were calibrated against a Schwarz vacuum thermopile which had been calibrated against a National Bureau of Standards lamp.

The irradiation vessels were quartz or Pyrex tubes with an internal diameter of 1 cm. A capillary tube was affixed to the bottom to provide stirring and aeration (or anoxia) by bubbling air (or nitrogen) through the suspension. The vessel used in the near UV photo-reactivation experiments was equipped with a water

jacket to maintain a temperature below the ambient. Cell suspensions, during irradiation, were maintained at either 0 to 2°C or 24 to 26°C.

Dose-survival curves are approximately described by the equation:

$$S = 1 - (1 - e^{-kD})^n,$$

where S is the surviving fraction, D is the dose in $\text{erg mm}^{-2} \text{ sec}^{-1}$, k is the inactivation constant in units of $(\text{erg mm}^{-2})^{-1}$, and n is the "shoulder" constant or extrapolation value.

FIG. 219.—Dose-survival response of *E. coli* WP2, *E. coli* WP2 *her*⁻, and *E. coli* K12 AB2480 irradiated with 254-nm UV.

FIG. 220.—Dose-survival response of *E. coli* WP2, *E. coli* WP2 *her*⁻, and *E. coli* K12 AB2480 irradiated with 365-nm UV.

PROGRESS REPORT

Dose-response curves were made for *E. coli* K12 AB2480, *E. coli* WP2 and *E. coli* WP2 her⁻ at three wavelengths; 254 nm (Figure 219), 365 nm (Figure 220), and 460 nm (Figure 221). These wave-

FIG. 221.—Dose-survival response of *E. coli* WP2, *E. coli* WP2 her⁻, and *E. coli* K12 AB2480 irradiated with 460 nm visible light.

lengths were chosen because they represent available bands in the far UV, near UV, and visible light spectrum. The pattern of sensitivity appeared to be qualitatively similar at the three wavelengths among these strains that differ in excision repair and recombination repair capability. It is significant that the curve shape of *E. coli* K12 AB2480 remained a simple exponential at all three wavelengths. *E. coli* WP2 retained the same shoulder size ($n = 200$ to 400) at 254 nm and 365 nm. At 460 nm, the data do not permit completion of the curve. The overall sensitivity changed by a factor of more than one million from 254 nm to 460 nm. *E. coli* WP2 her⁻ showed a small shoulder ($n = 1.5$ to 3.0) at 254 nm and 365 nm. At 460 nm, the shoulder size increased to an n of 150.

Although the sensitivity pattern is similar among the three strains at the three wavelength ranges, the relative spread of sensitivities among the three strains is greater at 254 nm, decreasing considerably at 365 nm and 460 nm. The relative energy levels required to obtain three log₁₀ cycles of inactivation of *E. coli* K12 AB2480 at the three wavelengths are extremely wide. For 254-nm UV, less than 2 erg mm⁻² are required for 0.001 survival. For 365-nm UV, approximately 5×10^5 erg mm⁻² and for 460-nm visible light approximately 1.5×10^8 erg mm⁻² are required to achieve 0.001 survival. This is a factor of 1×10^8 between 254 nm and 460 nm.

FIG. 222.—Dose-survival response and photoreactivation of *E. coli* K12 AB2480 irradiated with 365 nm UV at 1°C. Photoreactivation, 200 erg mm⁻² sec⁻¹.

FIG. 223.—Dose survival response and photoreactivation of *E. coli* K12 AB2480 irradiated with 365-nm UV at 25°C. Photoreactivation, 200 erg mm⁻² sec⁻¹.

The effect of photoreactivation on damage in *E. coli* K12 AB2480 caused by near UV (365 nm) is shown to be highly significant after inactivation at 1°C (Figure 222), but only a small effect is seen at 25°C (Figure 223). The damage is partially reversible with 30-min exposure to 430 ± 10 nm light at an irradiance of 200 erg mm⁻² sec⁻¹ after inactivation by 365-nm UV (1.5×10^3 erg mm⁻² sec⁻¹) at 1°C. The photoreactivable sector is approximately 0.5. Above the saturation irradiance for photoreactivation, the Q_{10} is 2.5.⁽⁷⁾ The time required for complete photoreactivation, at 25°C, above saturation irradiance, is 8 min.⁽⁷⁾ At 1°C, complete photoreactivation requires about 90 min. As inactivation at the highest irradiance available at 365 nm (1.5×10^3 erg mm⁻² sec⁻¹) requires approximately 10 min per log₁₀ cycle, it is evident that at 25°C photoreactivation occurring concomitantly with inactivation would be almost complete. Therefore, very little effect would occur on subsequent exposure to 430-nm light. However, inactivation by 365-nm UV at 1°C would result in much less concomitant photorestitution resulting in a much greater photoreactivation by 430 nm light. Even so, some photoreactivation does occur at 1°C, making it likely that the photoreactivable sector of 0.5 is an

underestimate of the damage that can be reversed by visible light. The photoreactivable sector in *E. coli* K12 AB2480 after inactivation by 254-nm UV is 0.82.

CONCLUSION

The greatly increased sensitivity of *E. coli* WP2 her⁻ and *E. coli* K12 AB2480 to 365-nm UV suggest that there is a major involvement of lesions that can be repaired by both the excision repair system and the recombination repair process in the inactivation of *E. coli* by near UV. The photoreversal of 365-nm damage implicates pyrimidine dimers in DNA as a major lethal lesion induced by this wavelength.

The nature of lethal lesions produced by 460-nm light is not established by these results. The increased sensitivity of *E. coli* K12 AB2480 to 460-nm light, a strain deficient in both excision repair and recombination repair, indicates that damage to DNA that can be repaired by normal strains occurs in this strain. These results do not eliminate membrane damage as a major component in the lethal effects of visible light, at least in certain strains.⁽⁸⁾

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LETHAL EFFECTS OF LONG WAVELENGTH ULTRAVIOLET AND VISIBLE LIGHT ON CELLS

John R. Lorenz and Robert B. Webb

PURPOSE AND METHODS

Lethal and mutagenic effects of long wavelength ultraviolet light (UV) and visible light, with no added sensitizers, have been reported and studied by many workers; this work has been thoroughly reviewed.⁽¹⁻³⁾ The applied work in this area has proved both interesting and fruitful, particularly in the area of human diseases associated with photosensitization (e.g., xeroderma pigmentosum, porphyria, erythema, and possibly, skin cancer).^(1, 3, 4) Recent basic research in this field has involved attempts to determine the mechanisms for photosensitized mutations^(5, 6) and lethality.⁽⁷⁻¹⁰⁾

Our study was undertaken to continue previous research on the determination of the chromophore, the lesions produced, and the role of DNA repair systems in the inactivation of *E. coli* by various wavelengths of long UV and visible light.⁽⁹⁾

Escherichia coli strains W2, WP2 her⁻, B, Bphr⁻, and K12 AB2480 were used in these studies. Stationary phase cultures were produced by streaking the required strain on nutrient agar (Difco) slants and aerobically incubating them for 48 hr at 37°C. A loop of surface growth was suspended in M9 salts solution without centrifugation and diluted approximately 1:10 in M9 medium to produce a suspension of approximately 10⁷ cells/ml for exposure to radiation. Log phase cultures were obtained directly from a Nephelostat,⁽¹¹⁾ which contained exponentially growing cells of the proper strain, typically growing at a concentration of 2 × 10⁷ ml, on M9 salts-glucose medium at 27°C. A typical growth rate would be 0.67 generations per hour. This suspension of growing cells typically was irradiated without washing or dilution. All suspensions were maintained below 27°C during irradiations; occasionally, if necessary, an air blower was used for cooling.

Typically, 0.1-ml samples were taken periodically during the irradiations, and 0.2 ml of appropriately diluted suspensions were spread on each of four nutrient agar plates. Survival is defined as the ability of a cell to generate a visible coliform colony after its incubation for 48 hr at 37°C or 72 hr at 25°C. Vessels for irradiation of cultures were quartz (for 254-nm UV) or Pyrex (for all other wavelengths) tubes with inside diameters of 1 cm. A capillary tube was fused into the bottom of the tubes for the passage of hydrated air for aerobic runs or prepurified N₂ (Mathe-

son Gas Products) for anaerobic runs. Unirradiated controls were periodically run to test for death in unirradiated cultures. No control series titer was altered significantly.

Short wavelength UV, at 254 nm, was supplied with a low pressure mercury lamp (Penray SC-11) with a filter to reduce the long UV and visible output, so that 95% of the output was at 254 nm. 313 and 365 nm were produced by the main line output of a 200-watt super pressure mercury lamp in a monochromator (Bausch and Lomb High Intensity). One source of visible light was a quartz-iodine incandescent lamp, using commercial slide projector optics (Sawyer 707Q). Two simple lenses converged the beam to a uniform spot of 1 cm². The most versatile source of long UV and visible light is a metal vapor arc lamp and power supply (General Electric) with a specially designed holder that produces an intense 1 cm² spot of broad spectrum light.⁽¹²⁾ With either visible light source, the desired wavelengths were passed, and the others were rejected by use of combinations of 2" by 2" interference or absorption filters (Baird-Atomic, Optics Technology, and Corning).

Irradiances below 1000 erg mm⁻² sec⁻¹ were measured with a Schwarz vacuum thermopile, standardized against a National Bureau of Standards lamp. The thermopile's output was measured with a Keithley 150B microvolt ammeter. Irradiances above 1000 erg mm⁻² sec⁻¹ were measured with a YSI-Kettering Model 65 radiometer calibrated against the Schwarz thermopile.

PROGRESS REPORT

Oxygen definitely appears necessary for damage to *E. coli* at 365 nm. Doses that produce survival of 10⁻⁵ with air bubbling, typically produce less than 50% lethality when the cells are bubbled with nitrogen.⁽⁷⁾

The excision-repair system appears to readily reverse damage to cells caused by 365-nm irradiation. *E. coli* WP2 her⁻, which differs from the wild type WP2 only in the lack of the enzyme responsible for initiation of excision repair, is several times more sensitive than WP2.^(7, 9) Liquid holding recovery, a phenomenon presumably caused by excision repair during extended holding of irradiated suspensions in tubes before they are plated, has been demonstrated in survival curves of log phase cultures of *E. coli* B r by Peak.⁽¹⁰⁾ Extensive trials to detect liquid holding re-

covery after 365-nm UV irradiation of stationary phase *E. coli* WP2 have failed in this laboratory. Peak also found an unexplained sensitization of log phase *E. coli* WP2 and B/r to 365-nm irradiation provided by acriflavine and caffeine.^(10, 13)

The recombination repair system, which also repairs DNA damage, appears to repair damage caused by 365-nm irradiation. Stationary phase cultures of *E. coli* strain K12AB2480, a mutant that cannot accomplish either excision or recombination repair, are much more sensitive to inactivation than stationary phase *E. coli* WP2 *her*⁻, which is only defective in excision repair.⁽¹⁴⁾

The third type of repair system that repairs damage to DNA, photoreactivation, appears to be active in repairing damage caused by 365-nm irradiation. The 365-nm UV, which inactivates cells, should simultaneously photoreactivate them, because 365-nm light is well within the wavelength range that produces photoreactivation (330 to 440 nm). Results seem to indicate, however, that a very small amount of photoreactivation will take place in *E. coli* WP2 *her*⁻ that has been previously inactivated with 365-nm light.

FIG. 224. -Survival of stationary phase *E. coli* strains B (wild type) and Bphr⁻ (a photoreactivationless mutant) after irradiation with 365-nm light.

While the effect is not large, in virtually every case survival was slightly higher in samples that were photoreactivated after irradiation than in those held and plated directly.

The effect is much more pronounced when stationary phase cultures of *E. coli* B and Bphr⁻ are inactivated, photoreactivated, and the effects of each compared. Bphr⁻ differs from the wild type B in that it appears incapable of photoreactivation. Survival curves, comparing the two, show that Bphr⁻ is two or three times more sensitive than B (Figure 224). Were it not that this amount of concurrent photoreactivation apparently takes place in all those strains which are capable of it, 365 nm would probably appear to be two or three times more lethal than it now appears to be.

Photoreactivation is the one DNA repair system left wholly intact in *E. coli* K12AB2480. These cells have considerably greater survival after inactivation with 365-nm light at 1°C when they are illuminated afterward with 430-nm visible light.⁽¹⁴⁾

Lethality with Visible Light

Most of the damage produced by broad spectrum visible light (390 to 750 nm) and narrow band 460-nm light appears to be oxygen dependent. Doses of 390 to 750 nm visible light that produce a survival value of 10^{-1} in aerated suspensions of *E. coli* WP2 *her*⁻, produce survival values greater than 0.9 in anoxic suspensions.

The excision-repair system appears to repair at least some of the damage produced by 390- to 750-nm and 460-nm visible light. Stationary phase *E. coli* WP2 *her*⁻ (excision-repair deficient) is slightly, but persistently, more sensitive to 390- to 750-nm irradiation than the repair sufficient strain *E. coli* WP2. Stationary phase WP2 *her*⁻ is approximately ten times more sensitive than stationary phase WP2 when it is irradiated with 460-nm light. In response to the same irradiation, log phase WP2 and WP2 *her*⁻ showed approximately equal sensitivities; both were approximately as sensitive as the previously mentioned stationary phase WP2 *her*⁻. When resistant stationary phase WP2 cells were also plated on nutrient agar plates with added acriflavine (British Drug Houses, Ltd., final concentration 5 $\mu\text{g/ml}$), they proved to be about ten times as sensitive, falling into the same range of sensitivities as stationary phase WP2 *her*⁻ and log phase WP2 and WP2 *her*⁻ (Figure 225). Acridine dyes (e.g., acriflavine) are known to inhibit the excision repair system.⁽¹³⁾

Recombination repair appears to reverse damage produced at 460 nm. At this wavelength, stationary phase *E. coli* K12AB2480 is considerably more sen-

FIG. 225. Survival of various strains of *Escherichia coli* after irradiation with 460 nm visible light. Bandwidth at half peak is 20 nm. Typical incident irradiance is 4×10^4 erg mm⁻² sec⁻¹.

sitive than stationary phase *E. coli* WP2 her⁻. Also, the K12AB2480 survival curve has no shoulder, as has the stationary phase WP2 her⁻. These strains essentially differ in that K12AB2480 cannot accomplish recombination repair, while WP2 her⁻ can (Figure 225).

It is not known whether damage produced by visible light wavelengths can be repaired with the photoreactivation system. One problem encountered is similar to that at 365 nm: visible wavelengths that are within the range effective for photoreactivation (about 330 to 440 nm) should photoreactivate the cells concomitantly with the inactivation produced. One approach will be to attempt to photoreactivate damage produced by visible wavelengths that are ineffective in photoreactivation, i.e., above 460 nm. Another approach which will be utilized uses the comparison between lethality of a wild type and its photoreactivationless mutant.

Action Spectrum for Lethality

A preliminary action spectrum (Figure 226) has been obtained for lethality produced with stationary

phase cultures of *E. coli* WP2 her⁻. The wavelengths used are 230, 240, 250, 254, 260, 270, 280, 290, 300, 313, 365, 410, 460, 510, 500 to 750, and 390 to 750 nanometers. Lethality is measured in terms of the "inactivation constant," the slope of the final, linear portion of each survival curve. The unit involved is (erg mm⁻²)⁻¹. It can be seen that sensitivity peaks near 265 nm, and is relatively high, but is falling at 313 nm. Inactivation at these wavelengths parallels the direct absorption of each wavelength by the cell's DNA, with oxygen-independent formation of pyrimidine dimers.

No direct absorption by DNA has been demonstrated either for 365 nm or higher wavelengths. A large amount of the repairable damage produced by these wavelengths, however, can be indirectly shown to be DNA damage, by the use of various repair-deficient mutants. Furthermore, the damage at these longer wavelengths is, to a large extent, oxygen dependent, as shown by the dotted, anoxic curve in Figure 226. Thus, oxygen appears to be essential in the utilization of longer wavelength energy by a chromophore which, in turn, effects the DNA damage.

DNA damage at 254, 313, and 365 nm can be repaired by photoreactivation, a process that is quite specific for the repair of pyrimidine dimers.

FIG. 226.—Inactivation constant plotted against wavelength for inactivation of *Escherichia coli* strain WP2 her⁻ with aerobic and anaerobic (dotted) irradiation. Brackets indicate width of wavelength band used.

CONCLUSION

Repairable DNA damage of various repair-deficient mutants of *E. coli* was demonstrated for all wavelengths studied. At wavelengths of 365 nm and above, this damage is indirect, because no absorption of these wavelengths in DNA has been demonstrated. Most damage at these longer wavelengths is also oxygen dependent. The data are consistent with an oxygen-dependent mechanism in which an intermediate chromophore absorbs light at the longer wavelength, transferring the energy to DNA, forming repairable DNA damage.

The nature of the lesion or lesions produced is not known, but at least some of the repairable damage produced at 365 nm can be demonstrated by indirect means to be the production of pyrimidine dimers.

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A SEQUENTIAL REPAIR MODEL OF PHOTOREACTIVATION IN BACTERIA*

David J. G. Davies,† Sylvanus A. Tyler, and Robert B. Webb

Kinetics of photoreactivation were studied in *E. coli* WP2 her⁻, a strain deficient in dark repair. Cells in aqueous suspension were subjected to UV irradiation, then exposed to photoreactivating light for different periods. Survival curves, with samples at a minimum of six UV doses, were obtained at several periods of photoreactivation ranging from zero to maximum. The surviving fractions do not conform to a dose-reduction model, but instead, they fit a "sequential repair"

model that assumes as a limiting condition that the number of active enzyme molecules is small. The model used assumed: 1) a single enzyme molecule is active at any one time; and 2) inactivating events are nullified consecutively around the DNA molecule. The mathematics of the model is derived and presented. Photoreactivation is attributed to the action of two processes: 1) A photochemical process that is rate limiting below 1000 ergs mm⁻² sec⁻¹ was measured at a photoreactivating irradiance of 60 ergs mm⁻² sec⁻¹. This has a rate constant of 5×10^{-5} "events" erg⁻¹ mm⁻². 2) A dark process, measured at photoreactivating irradiances of 4000 and 6000 ergs mm⁻² sec⁻¹, has a rate constant of 2.2 "events" min⁻¹.

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HIGH INTENSITY VAPOR ARC LAMP FOR BIOLOGICAL RESEARCH

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PURPOSE AND METHODS

Lethal effects of long wavelength ultraviolet light and visible light without external sensitizers have recently been studied in various strains of *Escherichia coli* B and B/r.⁽¹⁻⁴⁾

Formerly, the most practical light source was a quartz-iodine lamp utilizing a commercial slide projector optics system. The output was filtered and converged into an intense 1 cm² spot. Sufficient light was provided to produce meaningful survival curves using the whole visible spectrum (390 to 750 nanometers). However, despite many modifications optimizing the output, the energy available was not adequate to obtain a bandwidth narrow enough to produce a meaningful biological action spectrum. It was impossible to continue these studies unless a more intense source of broad spectrum visible light was found.

This report describes a light source that provides approximately 10^6 erg mm⁻² sec⁻¹ over the broad spectrum visible range and permits the continuation of this line of research.

PROGRESS REPORT

A commercially available light source and power supply (General Electric Co., Nela Park, Cleveland, Ohio) was adapted for use in biological research. The lamp is a 300-watt metallic vapor arc unit,* designated Mare 300.⁽⁵⁾

Because the lamp was developed for commercial projection systems, a completely new lamp holder was designed that satisfies our experimental requirements and complies with the manufacturer's specifications for the lamp. Figure 227 illustrates the lamp and condensing lens mounting and the provisions for an adjustable filter holder assembly. An air blower (Model No. C3508, Dynacool Mfg. Co., Inc., Saugerties, New York), located within the enclosure, directs approximately 150 ft³ min of cool air from the inlet upward across both the front and rear surfaces of the lamp to ensure efficient lamp operation. An air particle filter is mounted in the inlet to minimize contamination by dust of the lamp and condensing lens. An adjustable aperture, covering the air inlet, controls both the air flow through the enclosure and the operating temperature.

The lamp is replaced simply by sliding it into a track above the blower, and its location is fixed hor-

FIG. 227 A high intensity vapor arc lamp

izontally by the track and vertically by its stops. Lamp focus adjustments are never required.

Standard 2" by 2" interference filters are easily inserted in direct line with the condensed light beam. The holder is adjustable to rigidly position one or several filters in combinations chosen by the user. The object to be irradiated, for example a bacterial culture, is positioned in the path of the condensed and filtered light beam.

The power supply is especially designed and supplied by the manufacturer for use in standard projectors; it is designated as Mare-300 LSU-1.† It is intended for installation in an enclosure and does not include mounting and wiring of the fuse panel and control circuits. The completed power supply, shown in Figure 227, provides electrical safety and adequate ventilation for the power supply components. Figure 228 is a schematic diagram of the control wiring. This circuit includes a series connected fan-lamp switch that ensures operation of the fan before the lamp ignites and continued operation when the lamp is disconnected. A resettable running-time meter accumulates the total number of hours each lamp is used. A complete set of mechanical prints of the system is available.

The lamp's broad band visible output (approximately 10^6 erg mm⁻² sec⁻¹ in a small spot) is a fac-

* Approximate cost: \$25 00.

† Approximate cost: \$260 00.

FIG. 228. —Schematic diagram of the control circuit

FIG. 229. —Comparison of measured irradiances

tor of 10 higher than the output of the previously used quartz-iodine light source. Figure 229 compares the output of the vapor arc lamp with a quartz-iodine lamp and direct sunlight over the wavelength range of 350 to 750 nm. The sunlight measurements were

made with a Schwarz vacuum thermopile (standardized against a National Bureau of Standards lamp) and a Keithley Model 150B microvolt ammeter. The lamp measurements were made on a standard 1 cm² spot with a YSI-Kettering Model 65 radiometer (standardized against the Schwarz thermopile). Ample energy is available from the vapor arc source to allow the use of relatively narrow half-band width (20 nm) interference filters. For example, an irradiance of $7 \times 10^4 \text{ erg mm}^{-2} \text{sec}^{-1}$ was obtained through an interference filter having a peak transmission of 55% at 490 nm and a band width at half-peak transmission of 20 nm. The filters used were Baird-Atomic Type B3 for 350 to 510 nm and Optics Technology "Varipass" (Set 60) for 600 to 750 nm, with a peak transmission that varied from 35% at 350 nm to 65% at 650 nm. Much of this energy is available in the long UV and blue visible range. The conventional quartz-iodine lamp has very little output in this region.

CONCLUSION

The vapor arc light source makes possible studies of lethality with near UV and visible light that were impossible in a reasonable exposure time with the quartz-iodine lamp. There is sufficient output to provide a fairly detailed action spectrum in the long UV and visible region; 4 to 5 cycles of bacterial killing are produced.

In addition to the versatility and high useful output provided at a small fraction of the price of a comparable, commercially available system, the lamp and power supply are simple and hence convenient to operate.

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AN X-RAY CRYSTALLOGRAPHIC STUDY OF THE STRUCTURE OF CONCAVALIN A

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PURPOSE AND METHODS

In a program started last year,⁽¹⁾ we are attempting to solve the X-ray crystallographic structure of concanavalin A (Con A). This protein, which was isolated from jack beans (*Conavalia ensiformis*) binds a number of polysaccharides, including glycogens, amylopectins, dextrans, and mannans.⁽²⁾ It is most active over a pH range of 5 to 7. Because Con A also binds the polysaccharides from the surfaces of erythrocytes of various species, it is classified as a phytohemagglutinin. It has been found to suppress the immune response of mice after skin allografts.⁽³⁾

Con A has a molecular weight of 71,000⁽⁴⁾ and exists as a tetramer which has four crystallographically equivalent subunits.⁽¹⁾ The tetramer can be split into monomers and dimers by 8 M urea, and also can be split into dimers at a pH of 2.2.⁽⁵⁾ Con A has been shown to contain two carbohydrate binding sites per tetramer by equilibrium dialysis measurements with methyl α -D-mannopyranoside and methyl α -D-glucopyranoside.⁽⁶⁾ Therefore, the binding site must be shared between two subunits.

Con A has been reported to contain two divalent cations per tetramer and has been isolated containing three different ions, calcium, manganese, and magnesium.⁽⁷⁾ Metal-free Con A is completely inactive,⁽⁸⁾ although nothing is currently known about what role the metal plays in the binding of the carbohydrate. In addition to any of the three ions previously mentioned, activity can be restored by introduction of cobalt or zinc.⁽⁹⁾ Studies using disaccharides containing various combinations of the mannosyl and glucosyl residues to inhibit dextran binding by Con A have shown that the configurations around C3, C4 and C6 in the hexose unit at the non-reducing end of the polysaccharide chain are predominant factors in its binding specificity.⁽¹²⁾

We are particularly interested in Con A because it has many properties which are similar to those found in immunoglobulins—for example, binding a variety of carbohydrates, and having two binding sites per molecule. It is thought, therefore, that determination of the three-dimensional structure of this molecule might give an insight into the functional structure of antibody molecules. We are also interested in conformational changes of the protein molecule which are likely to be induced by the binding of various carbohydrate molecules. Because the molecule is made up of identical subunits, it is an excellent model for

studying allosteric effects (conformational changes produced when the first binding site becomes occupied, altering the binding potential of the second binding site).

More generally, solving the crystallographic structure of a protein with these interesting properties should help to identify parameters necessary for predicting three-dimensional structure of proteins from amino acid sequences.

The method of solving protein structure involves the use of at least two heavy-atom derivatives that are isomorphous with the native protein. At present, we are involved in the search for suitable derivatives. This requires surveying a large number of heavy-atom compounds by diffusing them into previously prepared crystals.⁽¹⁰⁾ Heavy-atom derivatives with only slightly altered lattice parameters, but with substantial changes in diffracted intensities, will be used for further study.

PROGRESS REPORT

The Picker-IBM 1130 automated diffractometer system, based on ARCADE, Argonne Computer Aided Diffractometer Equipment,⁽¹¹⁾ has been completed and is now operating. We are using a Cu K α source of 40 kV and 16 mA. The data collection programs

FIG. 230 - A reproduction of an actual pen tracing of a two-theta scan of the 00 l row of a Con A crystal. The scan proceeded from 0 degrees (at right) to 45 degrees (left). The 0012 reflection is noted at about 12 degrees two theta. The arrow indicates a threefold increase in chart sensitivity at about 9 degrees two theta.

were written and modified by J. A. Scherer (Applied Mathematics Division). This system can collect as many as 2,000 reflections per day. Programs necessary for studies of macromolecules by X-ray crystallography are being written and modified.

Tests to date are encouraging. The crystal remained aligned when data were collected for several days, indicating that vibration produced by the instrument has little or no effect. The angle positioning is precise, and the reliability of motor drives is good. The resolving power of the instrument in regard to the two-theta angle is shown in Figure 230. The intensities are plotted versus two theta. The intensity returned to background levels between reflections. The quality of the crystals has been shown to be excellent for X-ray diffraction studies. Many reflections appear in the vicinity of $2\text{ \AA}$; an example, the 0042 reflection, is shown in Figure 230 at 42 degrees two theta. Figure 231 shows intensity plotted against the omega scan of the 600 reflection at two different slit widths. Line A shows the scan with a detector slit width of 0.04 degree, showing that the half intensity width was not more than 0.05 degree. The mosaic spread of a reflection indicates the degree of order within the crystalline lattice. Line B was another omega scan with the detector slit width of 0.2 degree (which will be the operating con-

FIG. 231. - Reproductions of actual pen tracings of two omega scans over the 600 reflection. Line A shows a scan with a detector slit width of 0.04 degree indicating the mosaic spread of the crystal (see text). Line B shows a scan with a detector slit of 0.2 degree used for actual data collection.

TABLE 92. HEAVY ATOM DERIVATIVES OF CONCANAVALIN

Derivative	Cell dimensions, * $\text{\AA}$		
	<i>a</i>	<i>b</i>	<i>c</i>
IrCl ₃	63.2	87.3	89.3
K ₂ PtCl ₆	63.4	87.2	89.2
Uranyl formate	63.0	86.8	89.0
IrNa ₂ Cl ₆	63.1	86.6	88.9
Thallous acetate	63.1	86.8	89.1
Native protein	63.2	87.1	89.1

* The standard deviations are 0.2 $\text{\AA}$ for *a* and 0.3 $\text{\AA}$ for the *b* and *c* dimensions.

dition for actual data collection) showing that the half-intensity width under these conditions is 0.17 degree. Under these conditions, this allowed a setting error of ± 0.04 degree from the true omega zero which produced a count of only 2% lower than the maximum count.

Table 92 shows a list of heavy atom derivatives which produce minor changes in the crystalline lattice parameters and appear promising from preliminary precession photographs. From this list, at least two must be found which we can use to solve the phase problem. The iridium trichloride, platinum hexachloride, uranyl formate, and iridium sodium chloride derivatives show distinct differences in intensities from both the native crystals and one another. The thallous acetate shows some changes in intensities from the native crystals, but they are relatively small.

CONCLUSIONS

The diffractometer system has been completed and can collect data with a high degree of precision, reliability, and speed. These crystals are very well suited for diffraction studies. Some reflections correspond to interplanar distances of at least $2.15\text{ \AA}$, and the mosaic spread is no larger than 0.05 degrees. A number of promising heavy-atom derivatives have been found for attempted phase solutions.

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A CHEMICAL STUDY OF THE BINDING SITES OF CONCAVALIN A

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PURPOSE AND METHODS

This is a new program designed to complement the X-ray diffraction studies of concanavalin A (Con A). It consists of studying the effects of various chemical modifications of Con A on its activity. For a brief discussion of properties of Con A, see the previous report. This study is directed toward determining the specificity of the binding sites, the types of bonds which are involved, (e.g., hydrogen bonds, salt linkages between oppositely charged groups, and van der Waals interactions), and the contribution of each to the binding of the polysaccharides. Attempts will be made to determine if the amino acid side chains which are modified are involved directly in the binding of the carbohydrate, or whether they alter the conformation of the molecule, changing the chemical potential of the binding sites. Because it appears there are two metal ions bound per tetramer,⁽¹⁾ and there have been shown to be two carbohydrate binding sites per tetramer,⁽²⁾ the role of the metal ion is of special interest. Some of the derivatives are to be designed so that they may be used directly in the crystallographic studies. The location of a chemically modified site in the electron density map of the protein molecule would be extremely valuable in fitting the polypeptide chain to such a map.

Some of the more likely possibilities of chemical

modifications to be surveyed are photooxidation and carboxymethylation of histidine (histidine is of particular interest because of possible involvement in chelating the divalent metal ion), reaction of N-bromosuccinimide and 2-hydroxy-5-nitrobenzylbromide with tryptophan, acetylation and iodination of tyrosine, esterification of the carboxyl side chains, and any one of a number of reactions with amino groups.

PROGRESS REPORT

We are surveying chemical modifications of Con A to find which ones affect binding activity. Studies under way currently are carboxymethylation of histidine residues and N-bromosuccinimide oxidation of tryptophan residues. These will be carried out in the presence and absence of metals and various carbohydrates. At least eleven of the twelve tryptophans per tetramer are oxidized by the N-bromosuccinimide in 70% acetic acid. At present, we do not know the number of tryptophans which must be oxidized to begin affecting the binding of carbohydrates.

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CHEMICAL AND ENZYMATIC CLEAVAGE OF CONCAVALIN A

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PURPOSE AND METHODS

As part of a program complementing the crystallographic studies (see preceding reports), initial steps have been taken to determine the amino acid sequence of concanavalin A (Con A). It is difficult to study this protein in a conventional way, primarily because the Con A molecule is resistant to the action of trypsin.⁽¹⁾ Trypsin is the most specific of proteolytic enzymes and one of the prime agents generally chosen for degradation of proteins.

The structural basis for the resistance of Con A to trypsin is an interesting problem in itself, and we are attempting to isolate peptides containing lysine and arginine residues (the usual sites of tryptic hydrolysis). This attempt is incorporated in a more comprehensive search for the best methods to cleave Con A. Thus far the protein has been enzymatically hydrolyzed with papain, chymotrypsin, and thermolysin, and chemically cleaved with cyanogen bromide.

In an accompanying report, Hardman et al. point out the similarities in the types of binding reactions in which Con A and animal immunoglobulins participate. While we had no reason to suspect that the two types of molecule would have the same reactivities toward enzymes, we initiated the degradative studies of Con A with papain, with which Porter⁽²⁾ first hydrolyzed rabbit immunoglobulins into large fragments. Chymotrypsin and thermolysin were used because the amino acid composition of Con A⁽³⁾ included relatively large numbers of residues considered susceptible to hydrolysis (e.g., tryptophan, tyrosine, phenylalanine, and leucine for chymotrypsin, and phenylalanine, leucine, and isoleucine for thermolysin). The published analysis⁽³⁾ also indicated the presence of 1.2 methionine residues per subunit, a number which can be extrapolated to two residues when oxidative losses are taken into account. We hope eventually to find a denaturing agent that will fully expose these residues to the solvent and thereby make them available for attack with cyanogen bromide. In the first trial we employed this reagent under conditions found acceptable for the Bence-Jones proteins.⁽⁴⁾

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PROGRESS REPORT

When Con A was treated with papain, the molecule was not broken into three large fragments like the rabbit antibodies. Instead, the hydrolysate contained an insoluble fraction and any small peptides, usually ranging from 2 to 5 residues in length. The chymotryptic digest consisted of a less complex mixture, but approximately half of the molecule was represented by insoluble peptides which could not be fractionated. Thermolysin also did not solubilize the entire Con A molecule, but the otherwise intractable "core" was soluble in 50% acetic acid. We may, therefore, be able to hydrolyze this core with an enzyme like pepsin, which is only active in acidic solutions.

Among the soluble peptides were several with arginine or lysine residues. The compositions of these peptides were not sufficiently unusual to explain the resistance to trypsin. The general explanation probably involves unfavorable orientations of the arginine and lysine side chains in the three-dimensional structure of the protein.

Con A is also resistant to cyanogen bromide at pH 2 when denaturing agents are absent. We succeeded in partially cleaving the protein in 5 *M* guanidine-HCl, but we have not as yet found a suitable system for the fractionation of the products.

CONCLUSIONS

Inaccessibility of sites for possible cleavage appears to be the major obstacle in the elucidation of the amino acid sequence of concanavalin A. The reasons for this inaccessibility will probably not become clear until the three-dimensional structure is determined by X-ray analysis.

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A NEW PROCEDURE TO COMPARE AMINO ACID SEQUENCES OF BENEC-JONES AND OTHER PROTEINS

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PURPOSE AND METHODS

As emphasized in previous reports in this series,^(1,2) myeloma proteins and 7S γ -globulins are composed of two light and two heavy chains linked by *interchain* disulfide bonds. In multiple myeloma, light chains produced in excess are often excreted into the urine. As urinary constituents, the light chains are named Bence-Jones proteins after their discoverer, and their presence is pathognomonic of multiple myeloma. The Bence-Jones proteins fall into two principal antigenic classes, K and L, the members of which are usually called κ - and λ -chains, respectively. The genetic control of these proteins is manifest in their amino acid sequences, no two of which are identical in either the light chain or heavy chain series. *Within* each species and antigenic class of light chain, however, the carboxyl halves of the molecules are relatively invariant, while the amino halves show wide variations. Even the carboxyl halves are different in members of other species or antigenic classes.

Because of these substantial differences, it is difficult to compare the sequences in any systematic way merely by inspection. We previously devised two-dimensional projections called "helical wheels" to represent sequences in helical segments of proteins,⁽³⁾ but Bence-Jones proteins appear to be largely nonhelical in conformation. The key feature in the successful application of the helical wheels is the observation that apolar side chains tend to be clustered in an $n, n \pm 3, n \pm 4$ distribution in helical segments. Because the relative distributions of polar and apolar residues are of such structural importance, we employed Fisher's equations⁽⁴⁾ to study these distributions in the *sequence* of Bence-Jones and other proteins. Our approach represents a departure from the conventional use of the equations, which are generally applied in the prediction of hydrodynamic properties from overall amino acid *compositions*, rather than sequences.

Equations

In Fisher's approach, the polar groups are assumed to be external, while apolar side chains were assigned to internal positions. The summed molal volumes, V_e and V_i , for the polar (V_e) and apolar (V_i) residues were used to define the quantity, p , according to the equation $p = V_e/V_i$. The value of p was compared with p_s , which is equal to p in a spherical molecule:

$$p_s = \left(\frac{r^2}{(r-d)^2} \right)^3 - 1,$$

where r = radius of the sphere, and d = thickness of the external shell of polar residues; d is assumed to be 4 Å. As it deviates from 1.0, the ratio of p to p_s is a rough measure of the "asphericity" of the molecule and is approximately equal to the frictional ratio, f/f_0 , for most proteins with molecular weights up to 47,000. Values smaller than about 0.9–1.0 have been associated with proteins that tend to aggregate in solution. The effective p/p_s ratios of these aggregates approach or exceed 1.0. Insulin and the α - and β -chains of hemoglobin are examples of such proteins, whereas myoglobin ($p/p_s = 0.90$) exists as a monomer in solution.

Calculations

All calculations of p/p_s were performed with an IBM 1131 computer. Emphasis was placed on the Dil κ -chain and the Hul λ -chain,⁽⁵⁾ but the sequences of other human and murine κ -chains and human λ -chains⁽⁶⁾ were considered for comparison. Values of p/p_s were calculated as a function of chain length, and the results were plotted automatically by the computer.

TABLE 93. VALUES OF p/p_s FOR HUMAN AND MURINE LIGHT CHAINS

Protein	Entire molecule	Amino half	Carboxyl half	First quarter	Second quarter	Third quarter	Fourth quarter
Human κ							
Dil	1.20	0.76	0.88	0.52	0.44	0.37	0.88
Cum	1.20	0.72		0.41	0.53		
Ag	1.26	0.76		0.52	0.49		
Roy	1.14	0.67		0.45	0.39		
Murine κ							
41	1.31	0.76	1.00	0.49	0.52	0.35	1.18
70	1.13	0.58		0.32	0.42		
Human λ							
Hul	1.25	0.67	0.79	0.39	0.70	0.29	0.91
Bo	1.18	0.82		0.45	0.60		
Ha	1.13	0.75		0.49	0.43		
Sh	1.13	0.76		0.44	0.54		

The values were calculated with an IBM 1131 computer, used in conjunction with Fisher's equations.⁽⁴⁾ After computation of ratios for the entire proteins, the sequences were divided into halves and quarters, and the calculations were repeated. Because the carboxyl halves of molecules from each type and species are relatively invariant, the results for these segments are given for only one representative of each group.

Similar plots were constructed for sperm whale myoglobin and the α - and β -chains of hemoglobin.¹⁷⁾

To examine smaller segments, the sequences of Bence-Jones proteins were divided into halves and quarters before calculation of the p/p_s ratios.

PROGRESS REPORT

Values of p/p_s for the entire Bence-Jones molecules are presented in Table 93. The wide range of 1.13 to 1.31 for these values reflects the diversity in the overall compositions of the human and murine light chains. The fact that all ratios exceed 1.0 implies that the number of polar residues was more than adequate to cover an apolar center.

The plots of p/p_s against chain length in the three globins, Dil κ -chain, and the Hul λ -chain, are shown in Figure 232. The curve for myoglobin is distinctly higher than those for the two chains of hemoglobin, despite the fact that all three globins have similar three-dimensional structures. The differences in the plots for the κ - and λ -type Bence-Jones proteins are even less pronounced, a finding that indicates similar distributions of polar and apolar residues along the polypeptide chains. The curves also illustrate the importance of the chain length in the Bence-Jones proteins. A p/p_s ratio >1.0 was not achieved until 175 amino acid residues were included in the calculations.

The ratios of p/p_s for halves and quarters of the light chain molecules are listed in Table 93. These values were used to consider questions of homology of the light chains. In this approach, the results were examined for possible stable structures with ratios ≥ 1.0 .

FIG. 232. Changes in p/p_s ratios with increasing chain length. The curves for sperm whale myoglobin (Mb) and the α - (Hb α) and β -chains (Hb β) of human hemoglobin are given in the first panel. Plots for the Dil κ -chain and the Hul λ -chain are presented in the second panel.

Comparison of sequences suggests that the two halves of light chain molecules are probably homologous.¹⁶⁾ More tenuous arguments support an assumption of homology in all four quarters of the molecules. Direct evidence for the primordial existence of smaller, stable molecules of human light chains was absent, because the p/p_s values for both halves and all four quarters were below 1.0. Ratios for the "constant" carboxyl halves were closer to values for stable structures than those of the "variable" amino halves.

The amino halves of the murine κ -chains were in the same category, but the carboxyl halves did have ratios greater than 1.0. Surprisingly, the value also exceeded 1.0 in the fourth quarter. This quarter contains the interchain half-cystine residue, the structural feature most likely to be preserved in the evolutionary process.

CONCLUSIONS

The distributions of polar and apolar residues in the Bence-Jones proteins can be conveniently compared by application of Fisher's equations to amino acid sequences, as well as to compositions. Graphic displays of the results indicate that the light chains are closely similar in their distributions of polar and apolar residues, in spite of widely diverging sequences. The results also support the suggestion that the ancestral predecessor of the light chains may have been as small as one-quarter of the present molecules.

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A CRYSTALLOGRAPHIC INVESTIGATION OF THE Mcg L-TYPE BENICE-JONES PROTEIN

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PURPOSE AND METHODS

A crystallographic study of Bence-Jones proteins should provide direct information about the structure of light chains, when they are not influenced by interactions with heavy chains. Crystals of both the Bence-Jones protein and the parent myeloma protein (see accompanying report) were obtained from the same patient (Meg) by Dr. Harold F. Deutsch at the University of Wisconsin. Dr. Deutsch kindly provided us with samples of the two proteins, and at Argonne we succeeded in crystallizing them in forms suitable for X-ray diffraction studies.

While we are continuing to collaborate with Dr. Deutsch, we currently prepare our own samples of Bence-Jones protein from urine. After precipitation with 90% saturated ammonium sulfate, the protein was dissolved in 0.1 M Tris-HCl buffer, pH 8.0. The Bence-Jones protein was crystallized by dialysis against deionized water at 4°. For diffraction, once- or twice-crystallized protein was dissolved in the Tris buffer and dialyzed in capillary tubes at 20°. Suitable crystals formed in 2 to 4 weeks. Diffraction patterns were obtained with Supper precession cameras.

We previously have emphasized that Bence-Jones proteins may be isolated in two stable molecular forms, M-S-S-Cys and M-S-S-M, in which M is a polypeptide chain with a molecular weight of 23,000.⁽¹⁾ The distinction is structurally important because of the inter-chain disulfide bond and non-covalent interactions between subunits in the M-S-S-M molecules. Moreover, Bence-Jones proteins, consisting of mixtures of the two forms [e.g., the Dil κ -chain and the Gos λ -chain⁽²⁾], failed to crystallize, and homogeneity in regard to molecular species became a factor to consider in crystallization. Major attention, therefore, was focused on the identification of the molecular species and the assessment of purity of the Meg protein used in the preliminary crystallographic studies. The molecular species was identified by gel filtration at Argonne⁽¹⁾ and by centrifugal analysis by Dr. Deutsch at Madison. The assignment of the principal antigenic class and the simultaneous assessment of purity were performed by hydrolysis with carboxypeptidase A.⁽¹⁾

PROGRESS REPORT

The effluent curves for the fractionation of dissolved crystals and the supernate to the crystals are shown in

Figure 233. The column of Sephadex G-100 was calibrated with the Dil κ -chain and the Hul λ -chain.⁽¹⁾ Crystalline Meg protein migrated as a single component in the position assigned to the M-S-S-M type of molecule in the Dil and Hul proteins. This assignment is consistent with the value of 45,500 obtained for the molecular weight by Dr. Deutsch. The pattern for the supernate shows evidence of impurities lower in molecular weight than the principal component.

The absence of major impurities in the crystals was reflected in the products of hydrolysis with carboxypeptidase A. For example, 0.97 residue of serine, the C-terminal residue of human L-type, but not K-type molecule, was released per molecular weight of 23,000 from the sample of dissolved crystals, while amino acids from contaminants were liberated in quantities ≤ 0.12 residue per mole. These results were altered only slightly (serine recovery = 1.00; impurities ≤ 0.08 residue per mole) when the principal column component was treated with carboxypeptidase A. A higher level of contamination (up to 50% of the serine recovery) was indicated by the quantities and types of amino acids liberated from the components in the supernate.

A photograph of the crystals is shown in Figure 234. The crystals, which are colorless and bladed, are elongated along the *b* axis, and are bounded by {100}

FIG. 233. Fractionation of the Dil κ chain, the Hul λ -chain, and the Meg λ -chain on the same column of Sephadex G 100 at 4°, with 0.5 N NaCl as eluent. The two samples of Meg protein were 1) dissolved crystals (see panel c) and 2) the supernate from the suspension of crystals. (see panel d)

FIG. 234 Photograph of crystals of the Meg Bence Jones protein

FIG. 235 A 12 precession photograph of the hkl zone for a crystal of the Meg Bence Jones protein

and $\{001\}$ faces. The dimensions are as large as $0.2 \times 2.0 \times 0.5$ mm ($a \times b \times c$).

The crystals used in the diffraction experiments were fragile, but the reflections were not significantly diminished after 12 to 18 hr of exposure to the X-ray beam (Cu K α , 50 kV, 18 mA). A 12° precession photograph showing the hko zone is presented in Figure 235. Other photographs contained reflections that corresponded to interplanar spacings of 2.3 Å.

The mmm symmetry of the three-dimensional diffraction pattern indicated an orthorhombic unit cell. The following systematic extinctions were observed: for $h00$, $h = 2n + 1$; for $0k0$, $k = 2n + 1$. Therefore, the space group was unambiguously identified as $P2_12_12$ (No. 18 in Ref. 3).

The dimensions of the unit cell were $a = 72.6 \pm 0.2$; $b = 81.9 \pm 0.2$; and $c = 71.0 \pm 0.2$ Å, and the calculated volume was 422,000 Å³. The fractional volume, V_{soln} , of solvent in the crystal was estimated to be 0.46. The number of subunits of molecular weight 23,000 in the crystallographic asymmetric unit was

estimated to be two, corresponding to the M-S-S-M dimer.

CONCLUSIONS

A human L-type Bence-Jones protein (Mcg) was crystallized from water in a form suitable for X-ray diffraction studies. The protein was obtained in high purity by crystallization alone, and further purification by gel filtration was not considered necessary.

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A CRYSTALLOGRAPHIC INVESTIGATION OF THE Mcg MYELOMA PROTEIN

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PURPOSE AND METHODS

A crystallographic study of the Mcg myeloma protein should provide a model for considering the structural basis of antigen-binding in functional antibody molecules. By comparing these results with those for the Mcg Bence-Jones protein, we also hope to determine what modifications, if any, occur in the light chain structure as a result of its being incorporated into the larger IgG molecule. Preliminary crystallographic studies of another IgG protein (a "eryoglobulin") have been reported,⁽¹⁾ but the present work represents the first attempt to investigate the three-dimensional structures of both the myeloma and Bence-Jones proteins from the same patient.

"Eryoglobulins" precipitate even in the presence of the salt in the serum when the temperature is lowered to about 4° . The Mcg myeloma protein is a "euglobulin," which is soluble in salt solutions, but not in water.

To isolate the Mcg protein, solid ammonium sulfate was added to the serum to bring it to 37% saturation. The precipitate was dialyzed at 4° against 0.02 M phosphate buffer, pH 7.4. After centrifugation, the supernate was placed on a column of DEAE-cellulose, equilibrated with the same buffer. The eluate from the

column was dialyzed against water at 4° . The pellet from the centrifuged sample was dissolved at pH 8 in 0.1 M Tris buffer which was 0.15 M in NaCl. Crystals were obtained from both the column eluate and the dissolved pellet, but only those from the latter sample were suitable for diffraction.

The diffraction techniques were similar to those described for the Mcg Bence-Jones protein.

PROGRESS REPORT

The IgG protein crystallized as colorless prisms, elongated along the c axis and bounded by $\{110\}$ faces. The dimensions of the prisms were as large as $0.4 \times 0.4 \times 3.0$ mm ($a \times b \times c$). A photograph of the crystals is presented in Figure 236.

Precession photographs contained reflections corresponding to interplanar spacings of 4 Å. The mmm symmetry of the three-dimensional diffraction pattern indicated an orthorhombic unit cell. The following systematic extinctions were observed: for hkl , $h + k = 2n + 1$; for $00l$, $l = 2n + 1$. The space group was, therefore, unambiguously identified as $C222_1$ (No. 20 in Ref. 2). The dimensions of the unit cell were: $a = 88.0 \pm 0.3$; $b = 111.2 \pm 0.4$; and $c = 186.9 \pm 0.6$ Å; the calculated volume was 1,829,000 Å³. The

FIG. 236.—Crystal of Meg myeloma protein

fractional volume of solvent in the crystals was 0.58, a value within the normal range of 0.27 to 0.65 for proteins.⁽³⁾

There were four IgG molecules in the unit cell. The crystallographic asymmetric unit consisted of a half-molecule, i.e., one light and one heavy chain with a combined molecular weight of 75,000. Therefore, the two halves of each molecule were related by a twofold rotation axis.

Our results are similar to those obtained by Terry *et al.*⁽¹⁾ for cryoglobulins. For example, the fractional volume of solvent in the cryoglobulin crystals was also 0.58, and the asymmetric unit was a half-molecule of the same size. The space group (monoclinic) was different, and the crystals were substantially more sensitive to radiation damage than those of the Meg protein.

CONCLUSIONS

For the first time, both the myeloma and Bence-Jones proteins from the same patient (Meg) were crystallized in forms suitable for X-ray diffraction studies. Crystallographic similarities between our Meg "euglobulin" and the "cryoglobulins" suggest that the three-dimensional structures of the two classes of immunoglobulins may be comparable, in spite of substantial differences in their amino acid sequences.

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PREDICTION OF HELICAL SEGMENTS IN GLUCAGON

Marianne Schiffer and Allen B. Edmundson

PURPOSE AND METHODS

The use of "helical wheels" to predict which segments in a protein have helical potential was described in earlier communications.^(1,2) Three other methods to predict helical segments from amino acid sequences have been developed.

- 1) Prothero's method⁽³⁾ is based on the frequency of occurrence of certain residues in helical regions.
- 2) Low, Lovell, and Rudko⁽⁴⁾ studied di- through hexapeptide sequences in known proteins to see which are characteristic of helical and nonhelical regions.
- 3) Kotelchuck and Scheraga⁽⁵⁾ classify their residues as helix-making or helix-breaking. Their conclusions are based on energy calculations of short range interactions, as well as empirical criteria to obtain a better fit to the data.

For a number of proteins all methods lead to similar predictions, but there are some differences in the results. Like Prothero, we tend to predict more helices than are actually present in large molecules, while Low, Lovell and Rudko, and Kotelchuck and Scheraga under-predict. The methods have heretofore not been applied to molecules smaller than insulin, and we decided to examine the results for the peptide hormone, glucagon, which has 29 amino acid residues. The X-ray structure of this peptide is currently being investigated by Haugen and Lipscomb.⁽⁶⁾

PROGRESS REPORT

The predictions based on the different methods are presented in Table 94. The "wheels" for the two helical regions we predict are shown in Figure 237. These two helices have to be separate by our criteria, which include the necessity for hydrophobic residues to be located on *one* side of the wheel in an $n, n \pm 3, n + 4$ distribution. These residues, which are circled in Figure 237, form a hydrophobic arc. One continuous helix, encompassing residues 5 through 28, would require two hydrophobic arcs on opposite sides of the wheel, as is illustrated in Figure 238.

At the Eighth International Congress of Crystallography Lipscomb reported that glucagon is 75% helical, with two sections of helix. The C-terminal hexapeptide segment is not helical. Our results are in general agreement, although we do include five of the last six residues in the second helix predicted.

TABLE 94. COMPARISON OF PREDICTIONS OF α -HELICES IN GLUCAGON

Authors	Helical segments		% helix
Prothero ⁽³⁾	None		0
Low, Lovell, and Rudko ⁽⁴⁾	None		0
Kotelchuck and Scheraga ⁽⁵⁾	17-27		38
Schiffer and Edmundson ^{1,2}	5-16	17-28	83

RES. 17-28

CONCLUSION

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AMINO ACID SEQUENCING OF CATOSTOMUS CLARKI HEMOGLOBIN

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PURPOSE AND METHODS

Although the literature on serological poikilotherm "polymorphisms" is voluminous, theories that explain their origin and biological function are lacking or based on inadequate experimental results. Equally few attempts have been made to correlate "molecular polymorphisms" with environmental parameters.

Koehn recently described the electrophoretic heterogeneity of the hemoglobins from Catostomid fishes.⁽¹⁾ The number of unique electrophoretic bands varied from 8 to 12 per individual. To investigate the structural basis of these observations, the amino acid sequences of the alpha and beta chains of the hemoglobins from *Catostomus clarki* are being investigated. These results will be correlated with the genetic and evolutionary divergence of the hemoglobin loci as well as morphological and geological data and, we hope, with environmental parameters. A multivariate analy-

sis of the known hemoglobin sequences will be carried out to determine the degree of divergence.

The fish, *C. clarki*, were collected from the drainage canals near Phoenix, Arizona. Blood was obtained by cardiac puncture. The erythrocytes were washed several times, then lysed, and the hemoglobin was isolated by gel filtration on Sephadex G-100 and G-200. The heme group was removed by acid acetone precipitation of the chains.

The alpha and beta chains could not be separated by conventional procedures.⁽²⁻⁵⁾ Fractionation was achieved, however, by ascending chromatography on DEAE-Sephadex A-25.

After aminoethylation of the cysteine residues, each chain was hydrolyzed with trypsin for 24 hr. The tryptic peptides were purified by ion-exchange chromatography and high-voltage electrophoresis. Peptides were subjected to amino acid analysis and, if pure, to sequence analysis. The sequences were determined by modifications of the Edman phenyl-iso-thiocyanate procedure.^(6, 7)

PROGRESS REPORT

Partial separation of the intact hemoglobins has been accomplished by fractionation on DEAE-Sephadex, but complete separation has been achieved only by electrophoresis. Preliminary data from gel filtration indicates that all hemoglobins have the same molecular weight, which rules out polymerization of the hemoglobin units. However, ultracentrifugation is desirable to eliminate doubt of polymerization.

Excellent separation of the alpha and beta chains was accomplished on DEAE-Sephadex (Figure 239). There appear to be at least one alpha and two beta

FIG. 239. Separation of α - and β chains on DEAE Sephadex (A-25). The optical density is at 280 nm and is primarily a function of tryptophan and to a lesser extent tyrosine and phenylalanine residues. The optical density is plotted against the volume of effluent. The course of the NH_4HCO_3 gradient is indicated by the broken line.

FIG. 240. Separation on A-50-X10 resin of soluble tryptic peptides of the α -chain of *C. clarki* hemoglobin. Optical density at 570 nm of the ninhydrin reaction is plotted against the volume of effluent. The course of the pyridine and pH gradient is indicated by a broken line.

chains. The absorption of the alpha chain is smaller than that of the beta chains, because the alpha chain contains fewer tryptophan residues.

The elution profile for the fractionation of the tryptic peptides from the alpha chain is illustrated in Figure 240. Peptides represented on this chromatogram constitute about three-fourths of the alpha chain molecule. The remaining segments were in an insoluble fraction which is yet to be studied. Soluble peptides, representing approximately one-third of the beta chains, also have been isolated and purified.

From the compositions of the alpha chain peptides, it was evident that the sequence is similar to that of the carp alpha chain.⁽¹⁾ Fewer than 10% of the residues were involved in substitutions. No sequences of beta chains from fish have been published, but the peptides from globins of *C. clarki* appear to be substantially different from their mammalian counterparts. Several peptides from the beta chains of *C. clarki* appear to be more like the human delta or gamma chains than human beta chains. It is too soon, however, to draw conclusions about divergence.

CONCLUSIONS

Peptides from the alpha chains of *C. clarki* are less than 10% different from carp alpha chains. At present,

it appears that the "polymorphisms" result from different alpha and beta chain combinations.

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NEUTRON-GAMMA RESPONSE FOR LEAF AND COLEOPTILE GROWTH IN BARLEY

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PURPOSE AND METHODS

As part of a continuing program of cooperation with the International Atomic Energy Agency (I.A.E.A.), we have extended last year's studies of barley seedling growth⁽¹⁾ into new areas in an attempt to develop an international standard for seed irradiations. In our previous studies, we showed that the 5-day height of barley seedlings was reproducible to $\pm 3\%$, under the proper conditions and with appropriate statistical treatment, despite the observation that height distributions were strongly skewed toward short plants.⁽¹⁾ RBE values for 0.663 MeV neutrons or Janus neutrons were in the range 35 to 40, so that the system showed a high differential sensitivity to neutrons.

Using the same methods,⁽¹⁾ we now have examined the effects of growth periods and irradiation levels on the dose-effect curves and on the height distributions in an attempt to define the parameters that must be fixed if comparable results are to be obtained in dif-

ferent laboratories. Both leaf and coleoptile responses were considered because of the supposition that coleoptiles grow primarily by elongation, and leaves by cell division. Neutrons and gamma rays could affect these two processes differently, and an unhealthy coleoptile necessarily precludes a normal leaf. Thus, both structures were measured as a function of dose, radiation type and growth period, to determine their relative importance in the general economy of seedling growth and their eventual effect on subsequently determined RBE values.

PROGRESS REPORT

Seeds were equilibrated, irradiated, and planted as before.⁽¹⁾ After planting, the heights of the first leaf and of the coleoptile were measured separately at 3, 4, 5, 6, and 7 days of growth. Under the experimental conditions used, growth rate was constant for the first leaf and coleoptile grown from control and from irradiated seeds. Table 95 shows the results for selected neutron and selected ⁶⁰Co gamma irradiation doses. The results

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TABLE 95. GROWTH RATES OF NEUTRON AND GAMMA IRRADIATED LEAVES AND COLEOPTILES

Growth period, days	Coleoptiles, % of controls		Leaves, % of controls	
	0.663 MeV n	$^{60}\text{Co } \gamma$	0.663 MeV n	$^{60}\text{Co } \gamma$
3	75	66	50	37
4	77	64	52	43
5	76	64	48	39
6	76	63	43	35
Mean $\pm \sigma$	76.0 $\pm$ 0.7		48.3 $\pm$ 3.3	
	64.3 $\pm$ 1.1		38.5 $\pm$ 2.9	

Coleoptile values for seeds irradiated with 0.3 kRad neutrons or 20 kRad gammas; leaves with 0.9 kRad neutrons or 40 kRad gammas. Equally constant growth rates were obtained for all other dose values tried.

FIG. 241. Cumulative frequency plotted on probability scale against seedling leaf height at 6 days, following exposure of seeds to 663-MeV neutrons.

suggest that a single growing time would be representative of the radiation effects throughout the growing period studied, as the standard deviations were within 3% in both cases. Six days of growth were taken as representative.

Frequency distributions were made for each treatment at 6 days of growth. These are given in Figures 241–244 as cumulative probability against seedling

height. This type of plot allows several forms of analysis to be made:

(1) A straight line indicates a perfect, normal distribution. No plot showed this behavior, as noted previously.⁽¹¹⁾

(2) An abrupt change of slope indicates a secondary distribution. The unirradiated seeds showed one secondary distribution in the direction of an abnormal number of short seedlings. Such short seedlings could arise genetically or could be due to seed damage caused by harvesting or handling. High gamma doses introduced an additional secondary distribution skewed toward higher seedlings. This response would be expected from the known Z microdose distributions for γ rays.⁽¹²⁾ i.e., even at doses which seriously damaged most seedlings a few would escape essentially unscathed.

(3) The slopes of the distributions vary inversely with the standard deviations of the samples; the steeper the slope, the smaller the standard deviation of the sample. Gamma irradiation appeared to increase the deviation slightly, while neutrons tended to reduce the deviation. Again, this was to be expected on the basis of Z distributions and on the known increase of

FIG. 242. Cumulative frequency plotted on probability scale against seedling leaf height at 6 days, following exposure of seeds to ^{60}Co gamma rays.

FIG. 243. Cumulative frequency plotted on probability scale against seedling coleoptile height at 6 days, following exposure of seeds to 0.663 MeV neutrons.

sensitivity to small changes in seed hydration and oxygenation for gamma rays relative to neutrons.

(4) The horizontal displacement of each line shows the response of seedling growth to radiation dose. These are plotted in Figures 241–244.

(5) The gamma-irradiated seeds indicate there is a saturation dose between 50 and 60 kR. Up to 50 kR there remains a proportion of seeds which reach a height near the maximum height observed in the control sample; but beyond 60 kR seedlings are severely limited in height. This height at higher doses is reduced to a minimum, beyond which there is little change with increasing dose.

The seedling height, as percent of control height, was plotted on logarithmic scale (Figures 245 and 246). From these plots were derived the values given in Table 96. Fitting the logarithmic plot of dose response gives different extrapolation numbers for neutron and gamma radiation. This difference indicates that the RBE of 0.662 MeV neutrons is a function of percentage growth. Because it is inconvenient to treat RBE as a function, the ratio of D_{50} dose for gamma and neutron irradiation was selected by I.A.E.A. as the basis for RBE. For leaf growth this ratio is 41:1, and for coleoptile growth it is 24:1.

FIG. 244. Cumulative frequency plotted on probability scale against seedling coleoptile height at 6 days, following exposure of seeds to Co^{60} gamma rays.

FIG. 245. Correlation of leaf height at 6 days following gamma and neutron irradiation.

CONCLUSIONS

The ratio of $41/24 = 1.7$ between the RBE value for leaf and coleoptile agrees fairly well with the ratio found at 14 MeV,⁽³⁾ $11.0/5.1 = 2.2$. This suggests that mechanisms of radiation damage are similar at these

FIG. 246. Correlation of coleoptile height at 6 days following gamma and neutron irradiation.

TABLE 96. PARAMETERS FOR THE CURVES SHOWN IN FIGURES 245 AND 246

Test		N	kRad		
			D_0	$D_{q(10)}$	D_{37}
Leaf growth	Neutron	1.4	0.75	0.24	0.6
	Gamma	2.0	31	15	20
	Ratio γ/n	1.4	41	62	33.3
Coleoptile growth	Neutron	1.0	1.12	0	1.69
	Gamma	2.0	26	13	18
	Ratio γ/n	2.0	23.2		10.6

(*) D_q = intercept (kR) of extrapolated curve with 100%.

widely divergent energies, despite the increased average LET at 0.663 MeV, an increase reflected in the higher RBE values at the lower energy. The existence of different RBE values for leaf and coleoptile confirms

the suggestion that growth takes place by different mechanisms in the two structures. The RBE ratio of only two, however, suggests that much cell division must also occur in coleoptiles, so that the difference in growth mechanisms is not very large. Thus, coleoptile-versus-leaf comparisons provide only moderately sensitive systems for discriminating direct from indirect radiation damage. On the other hand, the high RBE values found, and their pattern of variation with neutron energy from 34 for Janus neutrons (mean energy 0.25 MeV) to 41 at 0.663 MeV, to 11 at 14 MeV, is in good agreement with the predictions of microdosimetry,⁽²⁾ suggesting that the barley-seedling system may be excellent for studies of the mechanism of RBE. In addition, the ease with which statistically large numbers of seeds can be irradiated and measured, their freedom from internal energy degradation, the moderate neutron doses required, and the fact that equivalent results can be obtained at any time from 3 to 7 days postirradiation recommends the barley-seedling system for general radiobiological studies, as well as for international intercomparisons. With the forthcoming availability of modified Janus fission facility, and of a Dynamitron monoenergetic facility, we will continue our studies of this system by examining the effects of dose rate and of neutron energies from 0.03 to 19.2 MeV on RBE and on relative leaf and coleoptile growth responses.

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EFFECTS OF ANTIBIOTICS ON DIFFERENTIATING CELLS. I. SENSITIVITY OF GRASSHOPPER SPERMATOGENESIS TO ACTINOMYCIN D

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PURPOSE AND METHODS

In the past, we employed ionizing radiation on cells in spermatogenesis (i.e., cells undergoing growth, division, and differentiation) to gain an insight into subcellular mechanisms of mitosis, meiosis, organelle differentiation, organelle production, and molecular synthesis.⁽¹⁾ Of the numerous antibiotics that affect protein synthesis and, hence, cell growth and development, two of the most widely used are actinomycin D and puromycin. They are most effective in cells undergoing growth and division.⁽²⁾ The effects of actinomycin D and puromycin are being investigated at present to elucidate these processes further with the expecta-

tion that the inhibitory effects of these antibiotics will yield indirect evidence of *normal* cell function. Particular attention has been given to the production of the plasma membrane at the cleavage furrow.⁽³⁾ Although no differences in the RNA-like granules related to this membrane synthesis have been noted, preliminary examination has suggested that many of the aberrations caused by radiation may also be induced by the antibiotics. We are dealing now with the experimental introduction of a chemical agent in the hope of tracing the aberration to its original cause and site within the cell. We appreciate that this focus entails recognition of many basic events in cell differentiation, including

FIG. 247. A spermatocyte shows an early form of myelin like figure in the smooth endoplasmic reticulum (ER)

the preferential binding sites of the antibiotics, abnormal molecular end products, ultrastructural changes, and the identification of abnormalities due to specific rather than general blockage of the cell's metabolism. Experiments using both puromycin and actinomycin D have been initiated, but to date, only the actinomycin D-treated tissue has been examined cytologically. Therefore, this preliminary report is restricted to effects of actinomycin D.

PROGRESS REPORT

The first problem has been to establish a suitable dose range of actinomycin D for the grasshopper and appropriate methods for the identification of cellular alteration due to this treatment. Last instar male grasshoppers, *Melanoplus differentialis*, were placed in groups of ten and maintained separately in small cages in the laboratory. Solutions of actinomycin D, 0.25 mg/ml, were prepared in Belar insect saline, and sub-

sequent serial dilutions of this solution to provide graded doses for injection were also made in the Belar. Each grasshopper received a 0.05-ml abdominal injection of a serial dilution, (the dose administered ranged from 0.0125 mg to 0.0000125 mg actinomycin D per animal). Two additional groups of ten grasshoppers each were kept as controls. Each grasshopper in one group received 0.05 ml Belar and the other group was untreated.

One grasshopper from each group was sacrificed at selected intervals of from 2 hr to 20 days post-injection. The testes were removed, dissected, and fixed for electron microscopy. Thick sections ($2\ \mu$) of this material, appropriately stained with toluidine blue,⁽¹⁾ were examined by light microscopy. The remaining grasshoppers during this period were observed to determine their survival and response to the treatments at the dose ranges used. The total procedure was repeated once.

FIG. 248. One daughter cell of a telophase pair shows an abnormal regrouping of chromosomes. Nuclear envelope (NE) formation has proceeded uninhibited, resulting in isolated nuclear fragments.

All animals that received the highest dose of actinomycin D (0.012 mg/ml) died by the third day post-injection. Approximately half of all of the grasshoppers in the other groups, including the Belar saline injected control group, were alive at 7 days, and very few deaths occurred thereafter, although the death check

was continued for a month. About one-quarter of the untreated controls died during these 30 days. Although the number of grasshoppers involved in this experiment is too small to be statistically significant, a trend was recognized that is a useful guide for future experiments.

Thick sections of testis, glutaraldehyde fixed⁽⁵⁾ and

FIG. 249. —A young spermatid contains several abnormal bodies of uncertain origin, but suggestive of certain cell organelles. The nearly tangential nuclear segment (N) appears normal, as does the nebenkern (NB) and tail filament bundle (T). The acrosome (A) is abnormally dark-staining within, although its size and capsule are normal. The limiting double membranes of the other dark structures (X) are very similar to transversely sectioned nuclear membranes as is the membrane of the nuclear-like upper body (X). Note the ribosomal particles on their outer membranes.

Epon embedded,¹⁶¹ were obtained from the same specimens that were sectioned and stained for electron microscopy. The most significant observations made by light microscopy on the thick sections of actinomycin D-treated material that were different from control sections included: 1) an excessive number of cells in diakinesis, and their dark-staining bivalents, in grasshoppers taken during the early time periods after injection; 2) the abnormal appearance of cysts in meiotic metaphase or anaphase, in which the cells had become a-synchronous and the chromosomes appeared thick, sticky, and unequally divided; 3) cysts of young spermatids in which numerous large nuclei occurred comparable to those of resting spermatocytes, suggesting a failure to either undergo or complete reduction-division; 4) a high incidence of supernumerary tail filaments in spermatids, frequently accompanied by either deep staining or "exploded" nebenkern bodies; and later, 5) a decreased number of cysts per follicle that contained maturing, elongating spermatids.

Similar sections in the electron microscope confirmed the observations made by light microscopy. A variety of ultrastructural abnormalities has been noted. The myelin-like figure, usually attributed to degenerating material, occurs frequently at all stages of development in the actinomycin D-injected material. This figure has been seen in close association with the mitochondria and endoplasmic reticulum (Figure 247). Within cysts of dividing cells, a disparity often occurs among daughter cells. In Figure 248, in one daughter cell, the chromosomes have failed to completely aggregate into the new chromatin mass, and the nuclear envelope has reformed around individual or clustered chromosomes. In contrast, its complementary cell, not illustrated, has proceeded normally in the telophase stage.

A persistent abnormal feature of the acrosome in actinomycin D-treated grasshoppers is its intense dark-stained appearance. In the spermatid in Figure 249, several aberrancies are evident. The dark acrosomal

FIG. 250 The distortion of the maturing spermatid nucleus is accompanied by supernumerary tail bundles (T) (four are noted) and by the inclusion of pinocytotic vesicles (PV).

FIG. 251. A cross section through the tail region of the developing spermatids shows multiple tail bundles (T). Some of these bundles have been disoriented and have lost the conventional $9 + 9 + 2$ configuration (arrows).

body lies between a normal-appearing nucleus and a membrane-limited granular mass that resembles the chromatin of spermatocytes. Other darker bodies surround the nebenkern. Their internal density is comparable to the acrosome, but their limiting membrane is more akin to the chromatin-like structure and to normal nuclear envelopes. These dark bodies could be interpreted, therefore, as various degenerate forms of abnormal nuclear bodies like those shown in Figure 248.

Distorted, maturing spermatids are numerous (Figure 250). Their loss of polarity is attended by supernumerary tail filament bundles, similar to those frequently observed in irradiated grasshoppers. These bundles typically retain their normal $9 + 9 + 2$ configuration, despite their multiplicity. In this actinomycin D-treated material, however, a partial or total loss of organization is not uncommon (Figure 251).

In Figure 252, two irregular structures are shown that have been observed in other material throughout

the study. At the cell periphery are a series of vesicles whose limiting membranes suggest a pinocytotic origin. These vesicles, whose number and size increase in the cytoplasm of early spermatids in actinomycin D-treated material, are absent in controls. Pinocytosis is not normally observed in grasshopper spermatogenesis. A stacked layer of *smooth* endoplasmic reticulum is located adjacent to the nuclear envelope, where one expects to find stacked *rough* endoplasmic reticulum. As this is the site of normal rough endoplasmic reticulum formation, this may represent an interference of ribosome synthesis in response to the actinomycin D treatment without the interruption of the membrane synthesis.

CONCLUSIONS

This program, of necessity, involves many facets of cell development. This preliminary report presents progress made in establishing dosage levels of actinomycin D and sacrifice times used to yield measureable

FIG. 252. The spermatocyte contains a group of pinocytotic vesicles (PV) underlying or attached to the plasma membrane. These are not typically associated with spermatogenic cells. Also, an unusual form, stacked endoplasmic reticulum (ER) devoid of ribosomal granules lies on the nuclear surface. Only granular endoplasmic reticulum is normally located at this particular site.

and identifiable disturbances of discrete cytological nature. In the future we hope to determine sites of vulnerability associated with deviation from normal organelle formation.

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LIPIDS IN THE LIVER CELL NUCLEUS

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PURPOSE AND METHODS

Lipoprotein preparations from isolated nuclei have been reported in a number of studies.⁽¹⁻⁴⁾ The possibility that they might be derived from the membranes of the nuclear envelope has been considered, but not definitely established. In subfractionation of isolated nuclei to yield nucleoplasmic and nucleolar fractions, the fate of the nuclear envelope has remained unknown.⁽⁵⁾

More recently, in autoradiographic experiments by Dr. F. Wassermann involving administration of radioactive palmitic acid to rats, grains were seen over the chromatin areas of the nuclei in electron microscope sections of liver and adipose tissue.⁽⁶⁾ They showed no tendency to be localized over the nuclear envelope, suggesting, therefore, that the nuclei absorb lipid from the cytoplasm.

The present work is aimed at elucidating the nature of the radioactive material revealed by autoradiography in the nuclei of the animals that received the labeled palmitic acid. It is aimed also at distinguishing between lipid associated with the nuclear membranes and lipid associated with the chromatin and the nucleoli. The first objective has been approached by using thin layer chromatography to isolate and then measure the radioactivity in the labeled palmitic and oleic acid liberated from the various lipids by acid hydrolysis. The second objective has been approached by studying the feasibility of removing the nuclear membranes from isolated nuclei and then isolating a chromatin fraction free of nucleolar contamination.

PROGRESS REPORT

Following intragastric administration, labeled palmitic acid appears in plasma chylomicrons predominantly in the form of triglyceride (>95%), which rapidly enters the liver without prior hydrolysis.⁽⁷⁻⁹⁾ In order to determine whether the radioactivity seen in the autoradiographs was still present in palmitic or oleic acid, and therefore legitimately to be considered in lipid, liver cell nuclei from animals that had received palmitic acid-9,10- H^3 24 hr previously were hydrolyzed with acid (6N HCl, 16 to 20 hr, at 105°C). The hydrolysate was evaporated to dryness under reduced pressure in an atmosphere of nitrogen; the residue was taken up in ethanol, and the dark solid was removed by filtration. The bulk of the nonlipid material was

precipitated by addition of $CHCl_3$, and the clear supernatant was subjected to thin layer chromatography on fiberglass paper impregnated with silica gel (Gilman SA; development with heptane—1.75% HAc). Lipid components were visualized as fluorescent spots under ultraviolet light after spraying with a solution containing rhodamine B. The fluorescent spot corresponding to palmitic and oleic acids (isolated together in this procedure) contained at least 85% of the total radioactivity in the isolated nuclei. It appears, therefore, that the grains seen in the autoradiographs do represent the presence of lipid in the nuclei. The nature of this material is being investigated further.

In the experiments dealing with removal of the nuclear membranes, the nuclei were isolated by the method of Blobel and Potter, and then washed in a Tris-potassium chloride-magnesium chloride buffer containing 0.5% Triton X100, at pH 7.5.⁽¹⁰⁾ Control preparations received the same treatment except that Triton X100 was omitted. After a subsequent wash in 0.34 M sucrose, the nuclei were pelleted in the centrifuge and fixed either with buffered potassium permanganate, or with glutaraldehyde followed by osmic acid. Dehydration, embedding in Epon, sectioning, and staining with uranyl acetate were done by conventional procedures.

In sections from glutaraldehyde-osmic acid fixed liver tissue, the outer membrane of the nuclear envelope is clearly evident (Figure 253). Visualization of the inner membrane is often obscured by the presence of perinuclear masses of dense chromatin, through which one sees channels leading to the pores in the nuclear envelope. These perinuclear masses of chromatin are seen also in isolated nuclei (Figures 254a and 255a). In nuclei sectioned nearly tangentially, it can be seen that the perinuclear dense chromatin actually comprises a continuous layer around the outside of the nucleus, in which the channels leading to the pores in the envelope appear as holes (Figure 255a). This perinuclear layer of organized chromatin may be responsible for the fact that the nuclei retain their shape even after ablation of the nuclear membranes by Triton X100. With permanganate fixation, the chromatin is distributed more evenly, and both the inner and outer membranes are clearly visible in the control nuclei (Figure 254a).

In the nuclei washed with Triton X100, fixation with glutaraldehyde and osmic acid reveals that the outer membrane certainly has been removed (Figure 255a).

* Deceased, June 16, 1969.

FIG. 253 Part of a rat liver cell illustrating the dense masses of chromatin immediately adjacent to the nuclear envelope. Glutinaldehyde-osmic acid fixation, 16,700 $\times$.

Occasionally, a faint line is seen at the periphery of a nucleus, but it is almost impossible to decide whether this represents a remnant of the inner membrane, or whether it is part of the perinuclear chromatin layer, which is still present. With permanganate fixation, the chromatin is more evenly distributed and except for an occasional faint suggestion of a peripheral line there is little indication that either nuclear membrane remains after the exposure to the Triton X100 medium (Figure 255b).

In order to separate the chromatin fraction from the nucleoli, the nuclei were subjected to brief sonication in a sucrose medium containing sufficient calcium ions to minimize disintegration of the nucleoli. The sonicate was then centrifuged over a cushion of 2.3 *M* sucrose, which retains the dispersed chromatin, but permits the nucleoli and the densest blocks of chromatin to pellet at the bottom of the tube.¹¹

In the chromatin preparation from nuclei subjected to brief sonication without prior removal of the nuclear membranes, we see many vesicles, some with single profiles, and some with concentric double profiles, one of which may have associated granules (Figure 256a). Apparently some are bounded by parts of both membranes of the nuclear envelope, and some by only one. The nuclear membranes have been whipped into a multitude of small vesicles, which are distributed

FIG. 254 Isolated liver cell nuclei not exposed to Triton X100. A, Glutinaldehyde-osmic acid fixation, 12,600 $\times$; B, permanganate fixation, 12,600 $\times$.

FIG. 255. Isolated liver cell nuclei washed with Triton X100 as described in text. A, Glutaraldehyde-osmic acid fixation, 9240 $\times$; B, permanganate fixation, 12,900 $\times$.

FIG. 256. Chromatin fraction from liver cell nuclei. A, Control preparation, 24,900 $\times$; B, preparation from nuclei previously exposed to Triton X100, 18,000 $\times$.

throughout the chromatin. Our results show that they do not separate from it even on prolonged centrifugation. In the chromatin from nuclei sonicated after removal of the membranes, such vesicles are extremely rare (Figure 256b). In this case, it appears that these arise from rare intranuclear vesicles, which are produced by invagination as a result of manipulation of the nuclei, and which then escape removal by the wash in Triton X100. Nucleolar contamination is very small in these preparations (Figure 256b).

Some detergents become firmly bound to proteins and this could complicate the study of proteins from nuclei exposed to Triton X100. However, detection of this contamination is facilitated in this case by the presence of a benzene ring in the structure (octylphenylpolyethoxyethanol). This produces a characteristic ultraviolet absorption spectrum (Figure 257), which is readily distinguished from that of protein. During acid hydrolysis, some cleavage evidently takes place at each of the ether linkages, as indicated by the pattern of spots produced in thin layer chromatography (Figure 258). Triton X100 scarcely moves from the origin; with the exception of the one at the solvent front, all of the fluorescent spots show the characteristic absorption peak. The hydrolysis products from the detergent do not interfere with the recognition and isolation of the spot containing the palmitic and oleic acids liberated by hydrolysis of the lipids (Figure 258).

FIG. 257. Ultraviolet absorption spectrum of Triton X100 in aqueous solution.

FIG. 258. Thin layer chromatography of palmitic acid (Samples 1 and 3) and the acid hydrolysis products of Triton X100 (Sample 2).

CONCLUSIONS

Previous autoradiographic experiments suggested the absorption of lipid by the cell nucleus. In confirmation, the present experiments have shown that at least 85% of the total radioactivity in the isolated nuclei is still present in the palmitic and oleic acids isolated by thin layer chromatography following exhaustive acid hydrolysis. The distribution of the labeled fatty acid among the various classes of lipids is still under investigation.

The autoradiographic observations mentioned above focussed attention on the long-standing need for studies aimed at distinguishing between lipids associated with the membranes of the nuclear envelope, and lipids associated with the chromatin and the nucleoli. The present report describes the exploration of a procedure for removing the nuclear membranes with Triton X100, and then using sonication and centrifugation to isolate a chromatin fraction free of nucleolar contamination.

It has been shown that if the nuclear membranes are not removed before sonication, they are whipped into a myriad of small vesicles, which are distributed throughout the chromatin.

Work is continuing on several aspects of this problem: the distribution of labeled palmitic acid among the isolated nuclear subfractions; the nature of the protein and lipid removed by Triton X100; the incorporation of labeled palmitic acid into chromatin during liver regeneration; and the application to our chromatin preparations of procedures previously used to isolate nuclear lipoproteins.

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FURTHER STUDIES ON THE ORIGIN OF AUXIN IN THE URINE OF THE MOUSE

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PURPOSE AND METHODS

Mammalian urine was one of the original sources for the isolation and characterization of the auxin indoleacetic acid (IAA). Urine has a relatively high concentration of auxin, an occurrence that is attributed largely to the ingestion of food that contains the hormone and to the metabolism of the gut flora.⁽¹⁾ However, free auxin is found in numerous animal organs,⁽¹⁻²⁾ and the auxin-forming enzyme complex, which converts tryptophan to IAA, also occurs in many vertebrates.⁽²⁾ The activity of the enzyme is particularly high in the liver, kidney, and gonads and is stimulated by exposure of the animal to ionizing radiation. The distribution of free auxin, and the presence of an endogenous biosynthetic pathway in the liver and kidney, suggests that IAA in urine might not necessarily be a product of the gut organisms and that the radiation response may be unrelated to these flora. Accordingly, we are examining the auxin economy of the germfree mouse.

In a previous report,⁽³⁾ we showed that IAA occurs in the urine of the germfree (GF) CRL:CD-1 (ICR)ax mouse, at concentrations not materially different from those in the urine of the conventional animal. We also found the tryptophan-IAA enzyme complex in cell-free homogenates of the liver and kidney of the GF mouse. Its specific activities in these organs were higher than those of the conventional mouse, a difference that could not be accounted for on the basis of gross protein concentration. In this report, we will present additional data on the activity of the tryptophan-IAA enzyme system and on the distribution of protein in the liver and kidney of GF and conventional animals. We will show that the GF animal can utilize tryptophan for the formation of IAA *in vivo*, and approximate the fraction of the IAA secreted in the urine that can be ascribed to ingested food. The methods used for the culture of GF animals, and the procedures for assay of enzyme activity and nitrogen distribution, were described previously.⁽³⁾

PROGRESS REPORT

There is only one locus of auxin activity in chromatograms of the acid fraction of urine from GF and conventional Swiss-Webster mice. This auxin activity corresponds to the Rf of labeled IAA in the presence of cosolutes, and there is no material difference in the amounts of urinary auxin between the GF and conventional animals. Thus, the observations made on the CRL:CD-1(ICR)ax mouse,⁽³⁾ hold for the Swiss-Webster mouse.

Additional assays of the tryptophan-IAA enzyme activities in hepatic and renal cell-free homogenates of the CD-1 mouse were run. The results are summarized in Table 97. Again, we find a relatively high titer of enzyme activity in the extracts of the germfree organs, both on tissue weight and protein bases. The coefficients of variation of enzyme titer range from 20 to 50%. The higher specific activities for the germfree preparations are not a consequence of lower protein levels: the TCA-precipitated nitrogen in the liver and kidney preparations from the germfree and conventional animals are not significantly different (Table 98). About two-thirds of the protein of the liver preparations resides in the "microsomal-soluble protein" fraction; for the kidney preparations, this fraction contains approximately half of the protein (Table 98).

The intracellular distribution of protein was examined further. Table 99 shows that there are no significant differences between the two types of animal in the protein contents of the nuclei-cell debris, the microsomal, or the "soluble" protein fractions. The mitochondrial fraction of the conventional liver has about one-third more protein than that of the germfree counterpart. Sedimentation analysis of the hepatic soluble fraction (Figure 259) demonstrates no qualitative or quantitative difference in gross protein moieties between the two types of animal.

We have shown that enzyme preparations of both the germfree and conventional animals can convert tryptophan to IAA *in vitro*. Does this conversion occur in the intact animal, viz., will the introduced

TABLE 97. TRYPTOPHAN INDOLEACETATE ENZYME ACTIVITIES OF HEPATIC AND RENAL CELL-FREE HOMOGENATES^(a)

	Liver		Kidney	
	$\mu\text{g IAA/g tissue hr}$	$\mu\text{g IAA/mg N/hr}$	$\mu\text{g IAA/g tissue hr}$	$\mu\text{g IAA/mg N/hr}$
Germfree	1.26 ± 0.26	0.067 ± 0.037	0.11 ± 0.13	0.031 ± 0.010
Conventional	1.23 ± 0.38	0.032 ± 0.012	0.12 ± 0.06	0.008 ± 0.004

^(a) $n = 9-10$.

tion of labeled tryptophan result in the appearance of labeled IAA in the urine? ¹⁴C-methylene labeled tryptophan was purified by paper chromatography and injected intraperitoneally into germfree and conventional mice [0.25 μCi (8.8 μg) in 0.5 ml physiological saline]. Pooled urine samples, collected 6 and 24 hr after injection, were partitioned and the acid fractions chromatographed, using methanol-*n*-butanol-acetic acid as the solvent system. Figure 260 shows the distribution of radioactivity on the chromatogram. Activity appears at the locus of IAA on chromatography of the urine extracts of both germfree and conventional mice. At 6 hr there is no material difference between the germfree and conventional animals in the amounts of IAA produced. The 24-hr samples, however, show significantly less activity at the IAA locus for the conventional animals. Moreover, considerable activity appears at the origin in the chromatograms of the extracts from the germfree animals, both at 6 and 24 hr. No activity was detected at this locus on the chromatograms of the extracts from conventional animals. These quantitative and qualitative disparities between the two animals reflect differences in competitive metabolic pathways for tryptophan or one of its derivatives.

It may be suggested from the preceding experiment (Fig. 260) that the urinary IAA arises, at least in part, from tryptophan. But part of the urinary auxin might come directly from ingested food. Is

free IAA absorbed from the alimentary tract and secreted in the urine? Because free IAA is readily inactivated in acid media, particularly in the presence of oxygen, labeled IAA dissolved in milk was intubated directly into the stomachs of both GF and conventional animals [2-¹⁴C-IAA, 15 nCi (~ 7 ng) in 1 ml milk]. Pooled urine samples were collected

TABLE 99. INTRACELLULAR DISTRIBUTION OF HEPATIC PROTEIN^a

Preparation	mg N/g tissue	
	Germfree	Conventional
Homogenate	21.1 $\pm$ 1.0	23.7 $\pm$ 0.78
Precipitate		
1,000 $\times$ <i>g</i> , 10 min	11.2 $\pm$ 0.37	10.8 $\pm$ 0.38
12,000 $\times$ <i>g</i> , 30 min	2.21 $\pm$ 0.16 ^(b)	3.01 $\pm$ 0.23 ^(b)
110,000 $\times$ <i>g</i> , 90 min	3.30 $\pm$ 0.14	3.02 $\pm$ 0.14
Supernatant		
110,000 $\times$ <i>g</i> , 90 min	1.20 $\pm$ 0.02	1.37 $\pm$ 0.06
Organ weights, g	1.66 $\pm$ 0.16	2.11 $\pm$ 0.15

^(a) $n = 4$.

^(b) $P < 0.05$, *t* test.

FIG. 259. Schlieren profiles during sedimentation of extracts from the livers of germfree and conventional animals. Livers perfused with physiological saline to remove interfering hemoglobin and then dispersed in 3 times their weight of 0.25 *M* sucrose in 0.04 Tris buffer, pH 7.3. Preparations were subjected to a preliminary centrifugation of 30,000 $\times$ *g* for 15 min (analytical ultracentrifugation by Lyle Bunville).

TABLE 98. TCA-PRECIPITATED NITROGEN IN HEPATIC AND RENAL PRIMARY (A) AND CELL-FREE (B) HOMOGENATES^(a)

	Liver				Kidney			
	A		B		A		B	
	<i>n</i>	mg N/g tissue	<i>n</i>	mg N/g tissue	<i>n</i>	mg N/g tissue	<i>n</i>	mg N/g tissue
Germfree	8	26.8 $\pm$ 0.97	14	18.8 $\pm$ 1.7	8	24.9 $\pm$ 0.66	9	13.4 $\pm$ 0.56
Conventional	6	26.5 $\pm$ 1.3	12	16.9 $\pm$ 0.40	7	25.2 $\pm$ 0.10	9	13.9 $\pm$ 1.1

^(a) 15,000 $\times$ *g*, 15 min.

at 4 and 24 hr after intubation, fractionated, and the acid fractions chromatographed. Figure 261 shows the distribution of radioactivity on the chromatograms. Activity appears only at the locus of IAA on the chromatograms, and there is no material difference between the activities of the GF and conventional animals, either at 4 or 24 hr. From these data, we suggest that IAA, in milk introduced into the stomach, is absorbed and excreted as IAA in both GF and conventional mice. On the basis of the total radioactivities measured, approximately 25% of the IAA introduced is eliminated, as such, within 24 hr.

If the animal can absorb and excrete the free IAA of food, what fraction of the urinary titer arises from this source? Mice of the size, age, and strain used, consume about 5 g of the autoclaved Lab-Blox supplied to them. By ether extraction, chromatography, and bioassay of the chromatogram, we find that the average amount of free IAA in one day's food consumption is, conservatively, about 25 ng. From the intubation experiment we have noted that ca. 25% of ingested IAA appears in the urine in 24 hr. On this basis, on the order of 6 ng of IAA in the urinary output for one day could be accounted for as originating in the free IAA of the food. However, the urinary excretion of IAA in one day, as determined by chromatographic resolution and bioassay, is ca. 2.5 μ g. Thus, the total amount of IAA excreted daily is at least 400 times that present as such in the food. We suggest that the ingested free IAA cannot account for the amount of IAA present in the urine of either the GF or conventional mouse. The difference must arise, therefore, by conversion of some IAA precursor in the animal.

If the mouse can absorb IAA intubated into the alimentary tract, what concentrations of auxin occur in the intestinal contents? We would anticipate that the intestine of the conventional animal contains free IAA, but had no basis for assuming a like occurrence in the GF mouse. Accordingly, intact intestines were removed from both types of animal and divided into three segments: the upper and lower small intestine, and upper large intestine. The contents of each segment were rapidly washed out with 0.01 *M* phosphate buffer, pH 8.0, acidified, extracted with ether at 3°C and the acid fraction of the extract chromatographed. The results of bioassays of the IAA locus on the chromatogram are summarized in Table 100. Typically, the caecum of the GF animal is enlarged. This is reflected by the 20-fold greater dry matter contained within this segment in the GF mouse. It is not unlikely that bacteria contribute to the relatively high concentration of IAA in the caecum of the conventional animal; however,

FIG. 260. Distribution of radioactivity on chromatograms of urine, acid fraction, following intraperitoneal injection of 3-¹⁴C-tryptophan. The arrows represent the *R_f* of IAA in admixture.

FIG. 261. Distribution of radioactivity on chromatograms of urine, acid fraction, following intubation of 2-¹⁴C-IAA.

TABLE 100. FREE IAA IN INTESTINAL CONTENTS OF GERMFREE AND CONVENTIONAL MICE

	Germfree			Conventional		
	Con- tent, dry wt., g	IAA, μ g/g	Total IAA, μ g	Con- tent, dry wt., g	IAA, μ g/g	Total IAA, μ g
Upper small intestine	0.340	0.62	0.21	0.200	0.88	0.18
Lower small intestine	0.860	≤ 0.012	≤ 0.01	0.430	≤ 0.023	≤ 0.01
Caecum	1.51	0.10	0.15	0.075	3.2	0.21
	2.7		0.37	0.70		0.43

the greater amount of matter within this segment in the GF mouse tends to equalize the total content of caecal IAA between the two types. The summed intestinal contents of IAA for the GF and conventional mouse are similar. These nonecumulative samplings, with IAA levels at least an order of magnitude greater than those found in a daily food intake, indicate that conversion of IAA precursors to IAA takes place in, or at least winds up in, the gut. Bacteria are not obligate to this process, though it is probable that the large concentration of IAA in the conventional caecum is of bacterial origin. We cannot explain why the ileum of the conventional animal, which presumably possesses a bacterial flora, has so low a level of IAA.

CONCLUSIONS

1. We find auxin, presumably IAA on the basis of chromatographic resolution and biological activity, in the urine in two strains of GF mice. Its concentration is not materially different from that of the urine of comparable conventional animals.

2. The tryptophan-to-IAA enzyme complex occurs and is active *in vitro* in enzyme preparations of the liver and kidney of GF mice. The activities in the GF organs equal or exceed those of conventional controls.

3. Labeled tryptophan introduced into the peritoneal cavity of both GF and conventional animals gives rise to labeled IAA in the urine. We suggest that both GF and conventional animals not only

can, but do convert the amino acid to the auxin. Endogenous bacteria are not obligate in the process.

4. The intubation of labeled IAA, as well as assays of the free auxin content of food, indicate that some of the urinary IAA could come from ingested free IAA and be absorbed as such from the alimentary tract. Quantitatively, however, ingestion of free IAA can account for only a small fraction of the amount that appears in the urine.

5. Assays of free IAA in the gut contents suggest that IAA formation takes place in the intestine, that bacteria are not mandatory for this production, and that it is probable that part of this IAA is absorbed and cleared renally. We suggest, therefore, that the major fraction of the urinary IAA of the mouse arises from endogenous enzymes intrinsic to that animal. The function of tryptophan as a substrate in this conversion is in no sense incompatible with a like function of other potential precursors of IAA, ingested or endogenous. We also suggest that microorganisms, *in situ* or invasive, may not be mandatory for the activation of the auxin-producing enzyme in the X-irradiated mouse.

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GROWTH AND CYTOLOGICAL EFFECTS OF LIGHT ON HAPLOPAPPUS IN SUSPENSION CULTURES

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PURPOSE AND METHODS

Previous studies here have shown that a high incidence of chromosome aberration occurs in pig kidney cells that were exposed to far-red light (ca. 730 nm). The aberrancies are similar to those caused by ionizing radiation. We were curious as to whether plant cells grown in the absence of light exhibited analogous photosensitivities. Accordingly, we are determining the effects of white and far-red light on the growth and cytology of plant cells grown in

suspension cultures. The present report describes several of the preliminary results.

Undifferentiating cells of *Haplopappus gracilis* ($2n = 4$) were grown under continuous agitation using a modification of the medium of Eriksson.⁽¹⁾ Inocula and suspensions for irradiation were made of mixtures of single cells and small colonies of less than 10 cells by preliminary filtration through 110- μ mesh nylon bolting cloth. (About two-thirds of the cells in the filtrate were single.) Exposures generally involved 5 ml of cell suspension containing from 500 to 2000 cells/ml of medium, spread by settling to a relatively uniform layer. White light was obtained

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TABLE 101. EFFECT OF WHITE LIGHT ON GROWTH OF *Haplopappus* CELLS^(a)

	Exposure, J·cm ⁻²	Fresh Wt., g	Dry Wt., g
Suspension	0	10.19	0.646
	1.4	9.89	0.608
	33	8.10 ^b	0.516 ^(c)
Agar	0	1.50	0.288
	1.4	3.67	0.237
	33	3.75	0.218

^(a) Of the 5-ml inoculum irradiated (2,500 cells), 1 ml was plated on agar and the remainder used for suspension culture. Growth period 3 weeks at 25 °C, five replicates.

^(b) Difference significant at the 2% level.

^(c) Difference significant at the 1% level.

from a tungsten halide incandescent lamp filtered by infrared reflecting glass. An interference filter, λ max. 730 nm, 15 nm HW blocked to "infinity", was added to obtain far-red light. Phased populations were obtained by keeping cell suspensions at 4°C for 6 hr before inoculation. For cell counts and cytological observation, cells were suspended in 1% sucrose solution containing 0.0025% Colcemid for 2 hr, fixed in Newcomer's reagent, and then stained with aceto-orcein. Permanent mounts were made of squash preparations. Cell counts were made by hema-

cytometer, generally after maceration of the stained cells in dilute nitric-chromic acid. *F* and *t* tests were used for variance analysis.

PROGRESS REPORT

Table 101 shows the effect of single exposures of white light on the subsequent growth of *Haplopappus* cells. Total cell weight, both in suspension and agar culture, is reduced by previous irradiation of the cells. To examine the effect of single exposures to white light on colony size and on ploidy, unphased inocula of filtered cultures were exposed to white light at four levels of incident energy. The influence of these exposures on cell number, type of colony and ploidy is shown in Table 102. Except in one instance, exposure to white light did not materially decrease the number of single cells produced in the suspension cultures. The exception was at the 10-joule level for the 7-day growth period of Experiment II. In all three experiments, there was actually an increase in the number of single cells and number of colonies in the cultures irradiated at the 0.1-joule level. However, the number of colonies formed after one week of growth was materially decreased by the greater exposures. This impairment appears to be transient, for there is no significant effect of illumination on colony number after a 14-day growth period. The decrease of colonies at the

TABLE 102. CELL NUMBER, COLONY AND PLOIDY DISTRIBUTION IN SUSPENSION CULTURES OF *Haplopappus* CELLS EXPOSED TO WHITE LIGHT^(a)

Experiment	Growth period, days	Exposure, (J·cm ⁻²)	Cells ml × 10 ⁻³	Colonies					Ploidy, %				
				Total 0.1 ml	% Distribution				<i>n</i>	2 <i>n</i>	4 <i>n</i>	5-32 <i>n</i>	
					2-10 cells	10-100 cells	100-500 cells	500-1000 cells					
I	7	0	192	406	57.7	31.0	10.1	1.2	1.8	81.2	8.0	9.0	
		0.1	209	455	60.7	28.1	9.8	1.1	2.0	81.2	7.8	8.0	
		1.0	177	268 ^(c)	72.1 ^(c)	13.4 ^(c)	12.1	2.2	2.0	70.8 ^(c)	12.6 ^(c)	14.6 ^(c)	
		10.0	180	284 ^(c)	69.4 ^(c)	14.2 ^(c)	14.4	2.1	5.8 ^(c)	47.6 ^(c)	28.2 ^(c)	18.4 ^(c)	
II	7	0	36	388	76.5	19.6	3.9	0	1.8	81.2	8.0	9.0	
		0.1	50 ^(c)	441 ^(c)	72.5	21.8	5.7	0	2.0	81.2	7.8	9.0	
		1.0	40 ^b	385	75.0	20.3	4.7	0	2.0	71.2 ^(c)	12.0 ^(c)	14.8 ^(c)	
		10.0	28 ^(c)	310 ^(c)	82.3 ^b	11.2 ^(c)	3.5	0	6.2 ^(c)	47.8 ^(c)	28.0 ^(c)	18.0 ^(c)	
III	14	0	338	3270	74.1	21.2	1.6	0.1	1.8	81.2	8.0	9.0	
		0.1	356	3360	74.4	23.8	1.6	0.2	1.8	81.0	8.0	9.2	
		1.0	334	3270	74.0	24.3	1.6	0.1	2.0	75.2 ^(c)	9.4	13.4 ^(c)	
		10.0	332	3230	74.0	24.4	1.5	0.1	4.6 ^(c)	50.0 ^(c)	29.2 ^(c)	16.2 ^(c)	

^(a) Five replicates per exposure. For number of cells, six samplings per replicate. For distribution of ploidy, 1000 cells scored per replicate.

^(b) Difference from control significant at the 5% level.

^(c) Difference from control significant at the 1% level.

TABLE 103. EFFECT OF FAR-RED LIGHT ON GROWTH OF *Haplopappus* CELLS^(a)

Growth period, weeks	Treatment	Colonies plate	Fresh Wt., g	Dry Wt., mg
1	Control	100	0.020	1.0
	Irradiated	60 ^b	0.012 ^(c)	0.6 ^(c)
2	Control	101	1.132	73
	Irradiated	69 ^(c)	0.781 ^(b)	50 ^(b)
3	Control	61	10.057	428
	Irradiated	45 ^(c)	8.126	367

^(a) Suspensions containing 500 cells/ml exposed to 50 millijoules·cm⁻², 1 ml plated out. Seven replicates.

^(b) Differences from control significant at the 1% level.

^(c) Differences from control significant at the 5% level.

TABLE 104. GROWTH AND PLOIDY IN PHASED SUSPENSION CULTURES OF *Haplopappus* CELLS EXPOSED TO FAR-RED LIGHT^a

Experiment	Growth period, days	Exposure, joules·cm ⁻²	Cells/ml × 10 ⁻³	n	2n	4n	5	32n	Aber- rant cells, %
I	10	0	381	3.4	80	7.8	9.0	3.8	
		0.01	403	5.0 ^b	74 ^(c)	10 ^(c)	12 ^(c)	6.0 ^(c)	
		0.1	408 ^(c)	5.5 ^(c)	63 ^(c)	19 ^(c)	13 ^(c)	15 ^(c)	
		1.0	392	10 ^(c)	12 ^(c)	21 ^(c)	24 ^(c)	20 ^(c)	
II	14	0	1510	3.4	79	8.0	9.0	3.8	
		0.01	1730 ^(c)	5.0 ^(b)	74 ^(c)	9.5 ^(c)	12 ^(c)	6.0 ^(c)	
		0.1	1650 ^(c)	6.0 ^(c)	63 ^(c)	17 ^(c)	13 ^(c)	14 ^(c)	
		1.0	1500	11 ^(c)	41 ^(c)	24 ^(c)	25 ^(c)	20 ^(c)	

^(a) Five replicates per exposure. For number of cells, six samplings per replicate. For distribution of ploidy and fraction of cells with chromosome aberrancies, 1000 cells scored per replicate.

^(b) Difference significant from control at 5% level.

^(c) Difference significant from control at 1% level.

TABLE 105. THE EFFECT OF FAR-RED LIGHT ON NUMBER OF ABLERRATIONS PER 100 METAPHASE CELLS^(a)

	Breaks	Exchanges	Haploids	Polyploids
Control	1.2	1.2	0	26
Irradiated	11.9	39.4	6.0	55

^(a) Cells exposed to 0.1 joules·cm⁻² 16 hr after subculture and scored approximately 10 hr after irradiation.

higher energy levels observed at 7 days was accompanied by a shift in the mean population per colony from the 10- to 100-cell class to the 2- to 10-cell category. Again, no material differences appeared at 14 days for the distribution of colony populations. Finally, ploidy within the populations was altered strikingly by white light at the 1- and 10-

joule level. There are highly significant increases in the frequency of polyploid cells, and at the highest energy a significant rise in haploid population.

Suspensions of cells were exposed to far-red light (50 millijoules·cm⁻²) and then plated out to determine the effect of this spectral region on growth (Table 103). Significant reductions were observed in both the number of colonies per plate and their fresh and dry weights. Cultures were then exposed to far-red light in the range of 0.01 to 1 joules·cm⁻², and grown in suspension for 10 or 14 days (Table 104). Again, an increase in cell number was observed at the lower exposures. However, far-red light at all levels progressively shifted the chromosome number from the diploid to the haploid and polyploid. Accompanying this shift of ploidy was a significant enhancement in the incidence of cells with chromosome aberrancies. The effect of far-red light in causing chromosome aberrations is characterized more explicitly by the analyses shown in Table 105.

As shown in Figure 262, chilling effectively phases cell division in *Haplopappus* suspensions. The cell cycle is ca. 20 hr. Eriksson⁽¹⁾ has shown that *Haplopappus* in suspension culture, with a cell cycle of 22 ± 3.8 hr, has the following stage durations of the cycle: *G*₁, 9.3; *S*, 6.4; *G*₂, 4.9; and *M*, 1.4 hr. Thus, we can infer that our exposures to far-red light were given at about the middle of the *S* period of DNA synthesis. For kidney cells, we have shown⁽²⁾ that this is the most sensitive period for the induction of chromosome aberrations by far-red light, and it is not unlikely that similar time-dependent differences in sensitivity hold for plant cells. It would be of interest to determine if synthesis of DNA in *Haplopappus* is impaired by far-red

Fig. 262. Phasing and cell cycle of *Haplopappus* cells in suspension culture. Cells were kept at 1° for 6 hr and then grown at 23.5°.

TABLE 106. MITOTIC INDICES IN SUSPENSION CULTURES OF *Haplopappus* CELLS EXPOSED TO 0.1 J·CM⁻² FAR-RED LIGHT 16 HR AFTER CHILLING^(a)

Growth period, hr	Mitotic index	
	Control	Irradiated
—	—	—
16	<0.01	<0.01
24	0.40	0.08
26	0.20	0.30
27	0.20	0.40
34	0.10	0.10

^(a) Each datum represents 1000 cells in mitosis.

light, an impairment demonstrated for kidney cells in monolayer culture.⁽³⁾ An action spectrum for the cytological responses of *Haplopappus* to illumination would be of aid in the identification of the photoreceptor involved.

Finally, irradiation of phased pig kidney cell populations at 8 delayed the time at which maximum aberration frequencies, induced by X radiation, were observed.⁽²⁾ From this, it could be inferred that far-red radiation causes a phase shift in the cell cycle. This is consistent with the observations of Wolff and Luippold^(4,5), who found that far-red light produced a phase delay in the cell division

cycles of the root tips of X irradiated *Vicia faba*. The influence of far-red light alone on the mitotic index at several times after irradiation of phased *Haplopappus* cells in suspension culture was, therefore, determined. The data of Table 106 suggest that exposure to far-red light also produces a phase delay in the cell cycle of *Haplopappus* that has not been exposed to X radiation.

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LIGHT AND RIBULOSE-1,5-DIPHOSPHATE CARBOXYLASE ACTIVITY IN ETIOLATED PLANTS

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PURPOSE AND METHODS

The enzyme ribulose-1,5-diphosphate carboxylase catalyzes the condensation of carbon dioxide with ribulose-1,5-diphosphate. This enzyme, which is located in the plastids of higher plants, is responsible for the conversion of carbon dioxide to carbohydrates. The enzyme comprises a large portion of the soluble protein in the plastid, the so-called "Fraction I" protein. Etiolated (dark-grown) corn, oat, and bean plants contain high levels of this enzyme in their leaves.

It was reported recently that a 3-min irradiation of etiolated corn leaves resulted in a 20- to 60% increase in the level of this enzyme over unirradiated controls.⁽¹⁾ The speed of this response, together with its reported insensitivity to short preincubations of the leaves with chloramphenicol, suggested that the

illumination might cause an activation of the enzyme in contrast to *de novo* synthesis. The experiments reported here were initiated to determine, first, whether this activation does occur rapidly after illumination, and, if so, what the spectral response characteristics are. And second, whether the change in activity could be best explained as a photoactivation of the enzyme as opposed to synthesis of new enzyme. A spectral response might indicate the nature of the photoreceptor involved and whether photoactivation of this plastid enzyme could participate in plant phototropism or photomorphogenesis.

Avena sativa cv. Victory I and *Phaseolus vulgaris* var. Red Kidney were grown in total darkness for varying periods up to eight days. Illumination, when required, was provided by a green⁽²⁾ safelight. Excised coleoptiles, or leaves, were floated on 2% sucrose on filter discs in 4-cm Petri dishes. Approximately 200 mg (fresh weight) of tissue were placed

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in each dish. Experimental treatments involved irradiation with either white light (quartz-halogen tungsten filament lamp) or monochromatic light obtained by insertion of an interference filter (660 nm, 15 nm H.W.) between the above source and the samples.

TABLE 107. RIBULOSE-1,5-DIPHOSPHATE CARBOXYLASE ACTIVITY IN ETIOLATED *Avena* LEAVES

Treatment	$\mu\text{moles CO}_2$ fixed mg protein hr
Unirradiated control	2.26 ± 0.20
45-sec white light, 900 mW cm^2	2.28 ± 0.20
45-sec white light, 900 mW cm^2 + 1 hr dark	2.11 ± 0.11

TABLE 108. DOSE RESPONSES OF RIBULOSE-1,5-DIPHOSPHATE CARBOXYLASE ACTIVITY UPON IRRADIATION OF ETIOLATED *Phaseolus* LEAVES WITH WHITE LIGHT

Irradiance, 100 $\mu\text{W} \cdot \text{cm}^{-2}$	$\mu\text{moles CO}_2$ fixed mg protein, hr	
	Experiment 1 (7 days old)	Experiment 2 (8 days old)
Unirradiated	2.38 ± 0.19	2.91 ± 0.37
$1 \times 10^3 \text{ ergs} \cdot \text{cm}^{-2}$ (1 sec)	2.61 ± 0.27	3.39 ± 0.23
$10 \times 10^3 \text{ ergs} \cdot \text{cm}^{-2}$ (10 sec)	2.62 ± 0.12	2.97 ± 0.12
$100 \times 10^3 \text{ ergs} \cdot \text{cm}^{-2}$ (100 sec)	2.60 ± 0.74	3.18 ± 0.49
Irradiance, 1.2 mW $\cdot \text{cm}^{-2}$		
Unirradiated	2.57 ± 0.49	
$0.72 \times 10^6 \text{ ergs} \cdot \text{cm}^{-2}$ (5 min)	2.78 ± 0.20	
$1.44 \times 10^6 \text{ ergs} \cdot \text{cm}^{-2}$ (10 min)	2.89 ± 0.39	
$2.16 \times 10^6 \text{ ergs} \cdot \text{cm}^{-2}$ (15 min)	2.87 ± 0.65	

TABLE 109. THE EFFECT OF RED LIGHT ON RIBULOSE-1,5-DIPHOSPHATE CARBOXYLASE ACTIVITY IN ETIOLATED *Phaseolus* LEAVES

Treatment	$\mu\text{moles CO}_2$ fixed mg protein hr	
	Experiment 1	Experiment 2 ^(a)
Unirradiated	4.83 ± 0.24	2.83 ± 0.33
5 min, 660 nm light, 70 $\mu\text{W} \cdot \text{cm}^2$	4.91 ± 0.22	2.81 ± 0.04

^(a) Lacking glutathione during extraction and incubation.

TABLE 110. THE EFFECT OF PROLONGED IRRADIATION WITH RED LIGHT ON RIBULOSE-1,5-DIPHOSPHATE CARBOXYLASE ACTIVITY IN ETIOLATED *Avena* COLEOPTILES

Treatment	$\mu\text{moles CO}_2$ fixed mg protein hr
Unirradiated	5.09 ± 0.24
11 hr, 660 nm light, 70 $\mu\text{W} \cdot \text{cm}^2$	5.88 ± 0.48

Extraction of the enzyme and all subsequent procedures were carried out at 0 to 2°C under the safelight. The samples were macerated in a glass mortar with a small amount of sand plus a solution of 1 part of 0.1 M Tris-SO₅ buffer, pH 7.4, to 1 part of 0.01 M glutathione. The extracts were centrifuged at $20,000 \times g$ for 20 min and the precipitates were discarded. An aliquot of the supernatant was diluted (1:10) with the extraction solution and used for assay of enzyme activity. The remainder of the supernatant was used for the determination of total protein colorimetrically by the Biuret procedure,⁽³⁾ with bovine serum albumin as a standard.

Ribulose-1,5-diphosphate carboxylase activity was measured by incubating a 0.5-ml reaction mixture of the following at 30°C: D-ribulose-1,5-diphosphate [barium salt of Sigma Chemical converted to the sodium salt by the method of Rabin and Trown⁽⁴⁾], 0.2 μmoles ; NaH¹⁴C¹⁸O₃, 0.5 mCi mM, 10 μCi ; 0.25 M Tris-Cl buffer, pH 8.0; 0.05 M MgCl₂; 0.001 M EDTA stock solutions, 0.1 ml; 0.025 M glutathione, 0.1 ml; enzyme protein. Reaction rates were linear to at least 750 μg of protein/ml of reaction mixture during incubation periods to 10 min. The reaction was stopped after 5 or 10 min by acidifying a 0.1 ml aliquot with 0.1 ml of 0.5 N HCl. 0.1 ml of this acidified mixture was placed on a 2-cm filter paper disk and dried overnight in a hood. The disks were then placed in scintillation vials, and 0.5 ml of water was added to elute the labeled products from the paper. Fifteen ml of scintillation fluid (4 g PPO, 0.05 g POPOP, 120 g naphthalene, dioxane to 1 liter) were added, and the samples were counted. ¹⁴C-toluene then was added as an internal standard to determine counting efficiency, which was usually 80 to 83%.

PROGRESS REPORT

The influence of illumination on activity of the leaf carboxylase was determined under the following experimental conditions: a, *Avena*, enzyme activity measured immediately and 1 hr after irradiation with white light (Table 107); b, *Phaseolus*, dosage-response to white light (Table 108); c, *Phaseolus*, exposed to red light for 5 min (Table 109); d, *Avena*, exposed to red light for 11 hr (Table 110).

The results presented in Tables 107 to 109, listing mean specific activities with their 95% confidence limits, show no significant effects of white or red light on activity of the carboxylase. These experiments involved exposures and assay within 1 hr after the beginning of irradiation. Because plastid development is initiated by the activation of phytochrome, the effect of prolonged irradiation with red light was

determined. A stimulation of activity, significant at the 5% level, took place (Table 110). An enhancement of activity of this carboxylase upon prolonged irradiation of etiolated leaves has been previously reported.⁽¹⁵⁻¹⁹⁾ On the basis of the present observations, we suggest that stimulation of activity of rubulose-1,5-diphosphate carboxylase by light is not a direct photoactivation but occurs as a consequence of the biosynthetic events accompanying photoinduced development of the plastid.

It has been suggested that the photomorphogenetic activations for which phytochrome is the photoacceptor are mediated via a change in redox potential, specifically an increase in the titer of free sulfhydryl groups.⁽¹⁰⁾ Thus the lack of immediate effect of light observed here could be accounted for as a masking of photoactivation by the presence of excess —SH in the medium used to disperse the enzyme. It is of interest, therefore, that use of a medium without glutathione before and during illumination does not result in detectable photoactivation (Table 109). We infer that a direct photoactivation of the enzyme was not replaced, and hence obscured, by chemoreductive activation in unirradiated tissues.

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RESPIRATION MEASUREMENTS DURING FLOWER INDUCTION IN XANTHIUM

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PURPOSE AND METHODS

The discovery of the circadian flowering rhythm in *Xanthium*⁽¹⁾ provides a unique tool for the investigation of the chemistry and physiology of the flowering process.

Of immediate interest was the possible direct correlation between respiratory metabolism in *Xanthium* and its observed cyclic flowering pattern. To facilitate these respiration measurements and more closely assess the behavior of the portion of the plant subject to floral induction and initiation, apical cuttings were employed. Prior to such use, it had been established that cuttings similar to those used in the respiration measurements responded as did whole plants with respect to a circadian flowering rhythm. Cuttings were made by excising plant tips a few centimeters below the three-fourths fully expanded

leaf at the sixth node. All leaves greater than 1 cm in length, except the one at the sixth node, were also excised and the cuttings were placed in quartz sand saturated with one-fifth strength Hoagland's nutrient solution. From the 8th through the 80th hr after excision, CO_2 evolution was assessed with an infrared gas analyzer, which served as the sensing element of a constant flow respirometer.⁽²⁾

PROGRESS REPORT

Maximum flowering in *Xanthium* cuttings and intact plants occurred during the 14th, 41st, and 68th hr after the start of the dark period. Maximums in respiration rates were observed during the 15th, 20th, 30th, 43rd, and 65th hr of the 72-hr experimental period. Whether the apparent coincidence of flowering maxima and respiration maxima is of direct biological significance is questionable, because the

plants kept under low-light conditions, which wholly inhibit flowering, exhibited respiration maxima of the same magnitude and at the same times as plants which were kept in complete darkness.

CONCLUSIONS

We conclude from these results that the flower-induction process has little effect on the overall plant respiration rate. This further suggests that though the physiological consequences of flower induction are substantial, large quantitative changes in plant me-

tabolism neither precedes, accompanies, nor immediately follows the initiation of this dark induced transition from the vegetative to the flowering state.

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THE SOURCES OF LEAD IN PERENNIAL RYEGRASS AND RADISHES*

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Experiments designed to assess the relative importance of air, water, and soil as sources of lead in perennial ryegrass and radishes showed that only air and soil were significant sources. Subsequent experiments reaffirmed that both grass and radish leaves

derived 2 to 3 μg of lead per gram dry weight from soil sources. Leaf lead levels in excess of this were derived from, and quantitatively related to, atmospheric lead concentrations. Lead levels in radish roots were less than or equal to soil-derived leaf lead levels. The lead concentration in this edible portion of the radish plant was apparently unaffected by variations in lead concentrations in either soil or air. Collectively, these data would not support a contention that the food of prehistoric man contained only a small fraction of the lead in the food of contemporary man, unless one assumes that prehistoric man assiduously eschewed plants as a food source.

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THE CONCENTRATION OF RADIUM, THORIUM, AND URANIUM BY TROPICAL ALGAE*

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Samples of twenty species of marine algae were collected between 1961 and 1968 from the coastal waters of Puerto Rico. In collection, emphasis was placed on areas related to the location of the reactor on the west coast of the island, before and after it went "critical." The algae were analyzed for total organic material, protein nitrogen and calcium, and radium-226, thorium-232, and uranium-238, naturally-occurring alpha particle emitters. From these analyses it is suggested that the concentration of radium, thorium, and uranium by tropical marine algae may be controlled by two mechanisms: 1) ion exchange or

coprecipitation of the ion with the calcium carbonate matrix, or 2) complex formation with either the protein nitrogen or some other component of the organic fraction.

Concentration of radium (and possibly thorium) appears to occur by both mechanisms, the dominant one depending upon the phyla. For the Rhodophyceae and the highly calcified Chlorophyceae it is the former, and for the Phaeophyceae the latter. Concentration of uranium occurs by the first mechanism. This difference in behavior is consistent with the chemical forms of the ions of these elements in seawater. The analytical data contribute to the characterization of the background level of radiation in the marine algae of the littoral waters of Puerto Rico. They also contribute to our understanding of mechanisms of biological concentration of actinide elements.

* Abstract of a paper to be published. A more detailed discussion appears in the Annual Report of the Radiological Physics Division, July 1968-June 1969 (ANL-7615).

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GROWTH AND DEVELOPMENT OF PLANTS IN COMPENSATED GRAVITATIONAL, MAGNETIC, AND ELECTRICAL FIELDS

OBSERVATIONS ON THE INTERACTION OF GRAVITY AND IONIZING RADIATION ON NUCLEI IN THE ROOTS OF VICIA FABA

Solon A. Gordon and Evelyn M. Buess

PURPOSE

As an index of the influence of gravity on radiation response, we are examining the effect of gravity compensation by clinostat on the components and morphology of the nucleus. We have found⁽¹⁾ that the mean DNA content of interphase nuclei in the roots of X-irradiated *Vicia faba* seedlings is not materially affected by gravity compensation. However, compensation shifted the temporal periodicities of nuclear volume and mitotic frequency and enhanced the incidence of micronuclei. Gravity compensation also increases the dimensional symmetry of the nucleus in the roots of unirradiated seedlings.⁽²⁾

The present report describes a continuance of the above investigation along the following two lines. First, the number of clinostats previously available did not permit a simultaneous test of the effect of gravity compensation on irradiated and unirradiated plants. Additional clinostats were constructed to allow tests of this matrix. Second, there are uncertainties about the validity of the single-wavelength technique for the microspectrophotometry of bound dye,^(3,4) the method we used for the estimation of DNA.⁽¹⁾ We have, therefore, shifted to the two-wavelength method^(3,4) for the photometry of the nucleus, with the following procedural extension: Two nuclear stains were used to differentiate between the root tips from two experimental treatments that were combined as a single squash preparation. This procedure was introduced because we have evidence⁽²⁾ that variations in pressure in the squash technique exert a strong influence on measured dimensions of the nucleus. We will present here several preliminary generalizations on the effects of gravity compensation and radiation on nuclear volume, DNA, and symmetry.

PROGRESS REPORT

Seeds of *Vicia faba* (Azores) were dusted with Ceresan-M and soaked in sterile distilled water at 4°C for 24 hr. After removal of the seed coat, the

seeds were inserted into cylindrical jars containing a hydrated mixture of vermiculite and Styrofoam as a supportive medium. The seeds were oriented within the medium so that the initial direction of the emerging root and shoot followed the longitudinal axis of the jar, the axis of rotation. The jars were clamped to multiple-unit clinostats of our design (Figure 263). One group was rotated at 2 rpm on horizontal axes of rotation, another was similarly rotated on vertical axes as a control. Plants were grown in the dark for 8 days at 27°C.

Eight days after planting, half of the compensated and half of the vertical, rotated seedlings were exposed *in situ* to 100 R X radiation (HVL 1.6 mm Cu), the remaining halves serving as unirradiated controls. Positions of the seedlings on the clinostats were chosen on the basis of previous phantom dosimetry so that exposures differed from one another by no more than 5%. Rotational motion was maintained during the eight days before, during, and after X irradiation. At various times after irradiation, three jars from each treatment were selected for analysis. Seedlings were not exposed to light after planting except for X irradiation and sampling, at which time green light at an irradiance level barely sufficient to allow manipulation was used. Only secondary roots were taken for analysis. These were treated with colchicine (0.2% in 1% sucrose) for 2 hr. They were then fixed in a modified Ford's reagent, cleared, and stained with either Feulgen (leuco-basic fuchsin) or toluidine blue. One-millimeter tips, cut under a dissection microscope, were taken for squash preparation and permanently mounted in Euparal. Two stains were used to identify tissues from two treatments squashed on the same slide. This procedure permitted the nuclei from two experimental treatments to be distinguished by color and yet be exposed to approximately the same squash pressure. Figure 264 shows the absorption spectra of interphase nuclei stained with the Feulgen and toluidine blue reagents. Absorbancies for the two-wavelength analy-

FIG. 263. —A "serpentine" clinostat. The device consists of a series of rotating shafts, one for each experimental chamber. All shafts rotate in synchrony since their drive pulleys are coupled to the motor by an endless belt.

FIG. 264. Microspectrophotometric absorption spectra of the interphase nucleus in secondary roots of *Vicia faba*. F, Feulgen stained; TB, toluidine blue.

sis were obtained at 560 and 497 nm for Feulgen-stained nuclei and 600 and 544 nm for those stained with toluidine blue.

For spectrophotometry and the determination of nuclear dimensions, horizontal scans were made across

the slide at the one-third, one-half, and two-thirds width. At each 1-mm position of the scan, the interphase nucleus closest to the center of the field was chosen. Two dimensions of the nucleus were measured—the major or maximum diameter and the minor diameter at the midpoint of, and normal to, the major diameter. (The ratio between the major and minor diameters was considered as an index of symmetry.) Scanning was continued until a total of 20 nuclei were obtained for each root tip. Five root tips were analyzed per experimental treatment at each sampling time. All slides were blind-coded to minimize bias.

The data on the effects of gravity treatment and X radiation on nuclear volume, DNA, and symmetry were subjected to analyses of variance. These analyses showed highly significant interactions between treatment and time for volume and DNA, but not for symmetry. An evaluation of these interactions will be presented in a forthcoming study. Here we will deal solely with the effects of gravity and radiation on the sampling sequence as a whole.

Because we have observed that nuclei become progressively more elongate with distance from the root tip, volumes were computed assuming that the nuclei were spheres, oblate spheroids, and prolate spheroids. Tables 111 and 112 show essentially no difference in volume ratios related to these geometric assumptions. It may be inferred that the shape of the nuclei in the terminal 1 mm of these secondary

TABLE 111. EFFECT OF X IRRADIATION ON THE RATIO OF INTERPHASE NUCLEAR VOLUMES OF GRAVITY-COMPENSATED TO VERTICAL, ROTATED PLANTS^a

		Sampling Time, hr											Mean $\frac{S}{(n=50)}$
		4	6	8	12	16	20	24	28	32	36		
Irradiated	A	1.20	1.03	1.52	1.86	1.20	1.08	1.19	1.36	1.31	1.40	1.32	0.056
	B	1.20	1.01	1.51	1.90	1.20	1.07	1.18	1.35	1.33	1.38	1.31	0.057
	C	1.20	1.08	1.54	1.75	1.20	1.09	1.21	1.38	1.26	1.46	1.32	0.057
Unirradiated	A	0.59	0.82	0.72	0.50	0.76	0.78	0.63	0.74	0.73	0.75	0.73	0.024
	B	0.60	0.81	0.73	0.49	0.76	0.78	0.61	0.73	0.72	0.74	0.73	0.024
	C	0.59	0.84	0.73	0.50	0.76	0.80	0.68	0.75	0.75	0.75	0.75	0.024

^a A, volume calculated as a sphere; B, as an oblate spheroid; C, as a prolate spheroid. Each datum in this and the following tables represents the mean ratio of 5 pairs of root tips, with 20 nuclei measured for each root tip.

TABLE 112. EFFECT OF GRAVITY COMPENSATION ON THE RATIO OF INTERPHASE NUCLEAR VOLUMES OF IRRADIATED TO UNIRRADIATED PLANTS

		Sampling Time, hr											Mean $\frac{S}{(n=50)}$
		4	6	8	12	16	20	24	28	32	36		
Compensated	A	1.66	1.87	0.95	1.85	1.54	1.72	1.89	2.05	1.70	1.28	1.65	0.067
	B	1.68	1.88	0.96	1.88	1.54	1.76	1.89	2.03	1.74	1.28	1.66	0.068
	C	1.61	1.84	0.93	1.77	1.52	1.64	1.90	2.09	1.59	1.29	1.62	0.066
Vertical, rotated	A	0.75	1.43	0.67	0.57	0.94	0.74	0.79	0.85	0.87	0.53	0.81	0.043
	B	0.75	1.47	0.67	0.58	0.95	0.76	0.78	0.84	0.88	0.53	0.82	0.045
	C	0.74	1.35	0.67	0.56	0.92	0.70	0.82	0.85	0.84	0.54	0.80	0.039

roots is approximately spherical. (We will comment further, below, on these symmetries.)

Each datum in Table 111 is a ratio describing the effect of gravity compensation. The ratios for unirradiated plants are consistently less than 1, with a mean of 0.73. Considered as a binomial distribution, the chance probability of this consistency is less than 0.1%. Or, with Student's *t* statistic as a criterion of a null hypothesis, the difference between 0.73 and 1 is highly significant ($P < 0.001$). Gravity compensation, alone, decreases the nuclear volumes. A similar decrease, with a like significance probability, is elicited by X radiation alone (Table 112, vertical, rotated mean of 0.81). Irradiation of the plant reverses completely the effect of compensation on nuclear volume (Table 111, 1.32 vs. 0.73, $P < 0.001$), and a compensation produces a similar action on the effect of X radiation on nuclear volume (Table 112, 1.65 vs. 0.81, $P < 0.001$). We conclude that there is a strong inverse interaction between the effects of gravity compensation and X rays on the volume of the nucleus in the tip cell of the secondary root.

Data on nuclear DNA are summarized in Tables

113 and 114. Gravity compensation, alone, has no significant effect on the mean DNA content of the nucleus (1.08 vs. 1, Table 113, $P \cong 0.1$). X radiation, alone, likewise has no material effect on mean DNA levels (1.05 vs. 1, Table 114, $P > 0.1$). The above lack of influence of compensation is not altered by irradiation (1.01 vs. 1.08, Table 113, $P > 0.1$), which substantiates our previous observations that the mean DNA content of interphase nuclei in X irradiated plants is not materially changed by compensation.⁽¹⁾ On the other hand, analysis of the data represented by Table 114 indicates that compensation increases the ratio between mean DNA level of irradiated to unirradiated plants by about 10% (1.18 vs. 1.05). This difference is nearly significant at the 5% level, but on the null hypothesis, 1.18 vs. 1 is highly significant, $P \cong 0.002$. The implication here of interaction might be considered equivocal in view of the indicated absence of interaction between gravity and radiation in Table 113.

The data on symmetry indices are summarized in Tables 115 and 116. We have shown that gravity compensation brings the symmetry index of the nucleus closer to 1 as compared to the erect, ro-

TABLE 113. EFFECT OF X IRRADIATION ON THE RATIO OF NUCLEAR DNA CONTENT OF GRAVITY-COMPENSATED TO VERTICAL, ROTATED PLANTS

	Sampling Time, hr											$S_{\bar{x}}$ ($n = 50$)
	4	6	8	12	16	20	24	28	32	36	Mean	
Irradiated	1.06	1.31	1.08	0.85	1.25	0.60	0.87	1.08	1.16	1.04	1.01	0.042
Unirradiated	1.01	0.94	0.92	1.30	1.07	1.59	1.33	1.34	0.59	0.78	1.08	0.044

TABLE 114. EFFECT OF GRAVITY COMPENSATION ON THE RATIO OF NUCLEAR DNA CONTENT OF IRRADIATED TO UNIRRADIATED PLANTS

	Sampling Time, hr											$S_{\bar{x}}$ ($n = 50$)
	4	6	8	12	16	20	24	28	32	36	Mean	
Compensated	1.05	1.39	1.55	1.18	1.22	0.65	0.82	0.89	1.70	1.32	1.18	0.053
Vertical, rotated	0.86	0.85	1.14	1.38	1.06	1.70	1.01	0.90	0.79	0.84	1.05	0.052

TABLE 115. EFFECT OF X IRRADIATION ON THE RATIO OF NUCLEAR SYMMETRY INDICES OF GRAVITY-COMPENSATED TO VERTICAL, ROTATED PLANTS

	Sampling Time, hr											$S_{\bar{x}}$ ($n = 50$)
	4	6	8	12	16	20	24	28	32	36	Mean	
Irradiated	1.03	0.94	0.99	1.11	0.99	0.97	0.98	0.99	1.05	0.93	1.00	0.013
Unirradiated	0.99	0.97	0.95	0.98	0.98	0.97	0.91	1.01	0.96	0.99	0.97	0.009

TABLE 116. EFFECT OF GRAVITY COMPENSATION ON THE RATIO OF NUCLEAR SYMMETRY INDICES OF IRRADIATED TO UNIRRADIATED PLANTS

	Sampling Time, hr											$S_{\bar{x}}$ ($n = 50$)
	4	6	8	12	16	20	24	28	32	36	Mean	
Compensated	1.05	1.04	1.04	1.05	1.02	1.07	1.00	0.95	1.11	1.00	1.03	0.011
Vertical, rotated	1.01	1.08	0.98	1.03	1.03	1.08	0.96	0.99	1.06	1.01	1.02	0.011

tated plant.⁽²⁾ This effect of compensation is reflected in the mean ratio of unirradiated compensated to vertical rotated plants (0.97, Table 115). The mean is significantly less than 1 ($P < 0.01$). X radiation, alone, raises, but not significantly, the ratio of symmetry indices (Table 116). Compensation adds to this effect of radiation, making the mean ratio of symmetry indices, 1.03, statistically different from 1 ($P \leq 0.01$). These differences are small, and perhaps not biologically significant. We suggest that the more spherical nuclei in 1-mm root tips than in the 2-mm tips used previously⁽²⁾ tend to minimize the effects of gravity compensation on nuclear symmetry.

CONCLUSION

The effects of gravity compensation and X radiation on the volume, DNA, and symmetry of the nuclei of *Vicia faba* roots were analyzed. Mean nuclear volumes were diminished both by gravity compensation and by X radiation. Irradiation of the plant reversed completely the effect of compensation on mean volumes, and compensation had a similar effect on the action of radiation. DNA contents of interphase nuclei (as indexed by microspectrophotometry of bound dye) were not materially affected by either radiation or compensation. However, compensation increased the mean DNA contents of the nuclei from

irradiated plants. We find again that nuclei are more spherical in gravity-compensated plants than in their erect controls. Though irradiation did not materially affect the nuclear symmetry, it did enhance significantly the effect of compensation. In summary, there is a strong interaction between the effects of gravity compensation and X rays on the volume of the nucleus, and an indication of interaction between these two treatments upon nuclear symmetry and DNA level.

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RECIPROCITY IN THE ACTIVATION OF GEOTROPISM OF OATS GROWN ON CLINOSTATS

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PURPOSE AND METHODS

The activation of geotropism of the normal plant has been shown to follow, within limits, a reciprocal relation between force, f , and time, t , $f \times t = k$ (constant).^(1,2) We define the time, t , at which activation of geotropism occurs in response to a constant force as the *presentation time*, the force-time product, k , as the *presentation stimulus*, and the response elicited by the presentation stimulus as the *presentation response*. We emphasize that this investigation is restricted to the study of reciprocity in the activation of geotropism.

Rutten-Pekelharing⁽¹⁾ demonstrated that reciprocity occurs in the geotropic presentation response of the oat coleoptile and garden-cress root. She found a k of 250-300 $g \cdot \text{sec}$, depending on temperature. More recently, Johnson⁽²⁾ calculated a k of 240 $g \cdot \text{sec}$ for oat seedlings grown in 22.5°C. Using a different approach, by testing various accelerations, we found a presentation response for continuous exposure (ca. 68 hr) to 0.0014 g , with a $f \cdot t$ product of approximately 340 $g \cdot \text{sec}$.⁽³⁾

The aim of the present study is to determine, first, if the reciprocity relation holds for the geotropism of oat coleoptiles grown in simulated "weightless" condition (i.e., under gravity compensation by horizontal clinostats of the serpentine type⁽⁴⁾). Second, if reciprocity does hold, is the product constant, k , similar to that for plants grown in normal orientation.

This study was carried out in two phases (for

methods of plant growth, gravity stimulation, and curvature measurement, see Reference 5). In the first phase, agar was used as the growth medium, and Johnson's⁽²⁾ extrapolation method was employed (presentation time extrapolated from the linear relationship, response to a constant force = $a + b \log \text{time}$). A total of seven accelerations between 0.08 and 3.0 g were tested. Each acceleration was repeated about five times, with an average total of 780 plants per acceleration. In the second phase, both sand and agar were used as growth media, and the stimulation times included very short intervals, i.e., 0.15 min. Under these conditions the response curve shows two distinct rate constants, a low rate for the short stimulation times, and a higher rate as stimulation time is lengthened (Figure 265). The intersection of the two rate segments (I and II) of the response curve was considered to be the presentation time. Curvature measurements were made from blind-coded shadowgraphs. All experiments were carried out at $27.2 \pm 1^\circ\text{C}$. All curves were fitted by the method of least squares.

PROGRESS REPORT

If the presentation responses of geotropism follow a reciprocity relationship between f and t , the product of these two variables will be a constant,

$$f \times t = k \quad (1)$$

or,

$$\log t = \log k + (-1) (\log f), \quad (2)$$

FIG. 265. Geotropic response curves of gravity-compensated oat coleoptiles; stimulus acceleration $0.2 g$.

TABLE 117. GEOTROPIC PRESENTATION STIMULI OF GRAVITY-COMPENSATED AND VERTICAL, ROTATED CONTROL COLEOPTILES OF OAT SEEDLINGS

Acceleration, g	Presentation stimulus, $g \cdot \text{sec}$		
	Gravity compensated	Vertical, rotated	Mean
0.08	88.3	81.1	84.7
0.2	97.2	102.0	99.8
0.4	76.4	66.4	71.2
0.6	116.0	105.0	110.0
0.8	122.0	85.6	104.0
1.0	131.0	118.0	124.0
3.0	68.4	121.0	94.5
Mean	98.8	97.0	98.3
$\sigma_{\bar{x}}$			± 6.5
Intercept ⁽¹⁾ $\pm \sigma_{\bar{x}}$	102.0 ± 1.1	96.9 ± 1.1	100.8 ± 1.1
Slope ⁽¹⁾ $\pm \sigma_{\bar{x}}$	-1.01 ± 0.09	-0.91 ± 0.07	-0.94 ± 0.06

⁽¹⁾ Derived from the least squares fit of log presentation time vs. log acceleration.

Equation (2) describes a linear function of the log-transform with intercept equal to the logarithm of k and a slope of -1 . We will show that the experimental data follow closely this relationship.

Using Johnson's extrapolation method, the presentation stimuli calculated for gravity-compensated and vertical, rotated (control) oat coleoptiles are not significantly different at the 0.05 level of Student's t distribution (Table 117). The data of the two treatments were, therefore, grouped and plotted in Figure 266 as log presentation time vs. log acceleration. The linearity and the slope, -0.94 with a standard error of ± 0.06 , indicate the compatibility of the

data with the criterion for reciprocity. The intercept, 100.8 ± 1.1 , is not significantly different from the mean presentation stimulus, $98.3 \pm 6.5 g \cdot \text{sec}$, derived from the data tabulated in Table 117. When the presentation stimuli of the vertical, rotated and stationary treatments were compared, 93.9 ± 20 and $85.7 \pm 18.1 g \cdot \text{sec}$, respectively, they also showed no significant difference. It may be inferred that gravity compensation does not alter the sensitivity of the physical perception of gravity by the oat coleoptile. Moreover, the linear relationships between presentation time and acceleration on log-log plots, with their slopes of close to -1 , indicate that the reciprocity rule is followed not only by normal plants^(1,2) but also by gravity-compensated and vertical, rotated oat seedlings.

The validity of using the extrapolation method for the determination of t could be criticized on the basis that there is no clear evidence for a discrete threshold in the "all or none" or "triggering" sense in plant geotropism.⁽³⁾ As pointed out under Methods, when the stimulation times were brief, a two-rate response curve (Figure 265, curve segments I & II; Table 118) was obtained. Table 118 shows that the slopes of segments I and II for the compensated plants are not significantly different from their comparable slopes for the vertical, rotated plants. (A similarity between the two gravity treatments is also shown in Table 119). The slopes (reflecting magnitude of response) for segment II are greater when

FIG. 266. Relationship between presentation time and acceleration (calculated) in the presentation response of geotropism of gravity-compensated, $\bullet$, and vertical, rotated control, $\times$, oat coleoptiles. Presentation times calculated by extrapolation. The regression line is derived from the means of the two treatments; $n = 780$ seedlings per acceleration.

agar is used as the growth medium. However, the coleoptiles grown in sand are significantly more sensitive in geotropic activation than those grown in agar (Table 119), the presentation stimulus being 61.3 ± 2.9 and 84.8 ± 6.3 g·sec, respectively. The f - t relationships for the two media are shown in Figure 267. The slopes are -0.96 ± 0.06 for sand, and -0.91 ± 0.08 for agar. These indicate once again the existence of reciprocity.

The intercepts tabulated in Table 119 are lower for the C than for the R treatment. They are significantly different when agar was the growth medium, indicating that gravity compensation might enhance the sensitivity of gravity perception by the oat coleoptile. We must point out, however, first, the experiments with agar as the growth medium

TABLE 118. MEAN SLOPES AND STANDARD ERRORS OF SEGMENTS I AND II OF RESPONSE CURVES⁽¹⁾

Treatment	Agar		Sand	
	Segment I	Segment II	Segment I	Segment II
Gravity compensated	0.96 ± 0.92	8.11 ± 1.53	0.51 ± 1.00	3.70 ± 1.15
Vertical, rotated	0.54 ± 0.62	8.12 ± 1.47	0.22 ± 0.67	3.29 ± 0.93

⁽¹⁾ See Figure 265.

TABLE 119. MEAN PRESENTATION STIMULI IN THE GEOTROPISM OF OAT SEEDLINGS GROWN IN AGAR AND SAND⁽¹⁾

Acceleration, g	Presentation stimulus, g·sec	
	Agar	Sand
0.07	67.8	
0.09		63.7
0.1		56.3
0.2	96.0	61.0
0.4		56.0
0.5		56.0
0.6	82.2	66.0
0.8	92.4	65.8
1.0		78.6
Mean	84.8	61.3
$\sigma_{\bar{x}}$	± 6.3	± 2.9
Intercept ^(b) + $\sigma_{\bar{x}}$	C	88.0 ± 1.1
	R	99.4 ± 1.3
Slope ^(b) ± σ_x	C	-0.89 ± 0.06
	R	-0.92 ± 0.15

^(a) Data for compensated and rotated plants grouped.

^(b) Derived from the least squares fit of log presentation time versus log acceleration. C, gravity compensation; R, vertical rotation.

FIG. 267. Relationship between presentation time and acceleration (calculated) in the geotropic presentation response of coleoptiles. Presentation times calculated by intersection. The regression lines are derived from the means of the compensated, ●, and rotated, ×, treatment for sand, and the mean, ○, of the two treatments for agar. Each acceleration was repeated ca. 4 times for sand and once for agar.

involved only one test at each acceleration, with only four accelerations tested. Second, there was no significant difference between the C and R treatment when sand was the medium, or when the extrapolation method (Table 118) with agar as the medium was used for the determination of presentation stimulus. Because of these considerations, we feel that the validity of the difference between the C and R treatment for agar in Table 119 is questionable.

CONCLUSION

Earlier works^(1,2) demonstrated the existence of reciprocity between presentation time and force in the presentation response of geotropism. We find this relationship also holds for plants grown under continuous gravity compensation. The sensitivity of gravity perception is not changed by the gravity treatment during growth, but is affected by the growth medium and temperature^(1,2). Seedlings grown in sand are more sensitive than those grown on agar to physical stimulation by gravity. However, the rate at which subsequent geotropic curvature develops is lower for seedlings grown in sand.

The presentation stimulus derived from the present study is less than those calculated by Rutten-Pekelharing⁽²⁾ and by Johnsson⁽²⁾. This could be a consequence of differences in temperature and medium. However, the presentation times derived from the two methods of approximation, extrapolation

and intersection, appear not to differ significantly. Although both methods show the existence of reciprocity in the presentation response of geotropism, the method of intersection appears to be more appropriate for the estimation of presentation stimuli, since it is based on observed data rather than on extrapolation.

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PARTICIPATION OF GOLGI APPARATUS IN GEOTROPISM

Jane Shen-Miller and Ray Hinchman

PURPOSE AND METHODS

Geotropism is a result of differences in cell elongation, differences in cell wall extension and accretion, in tissues proximal and distal to a gravity stimulus. Organelles that can be directly associated with expansion of the cell wall are the endoplasmic reticulum, the Golgi apparatus or dictyosomes, and the vesicles associated with both organelles.⁽¹⁾ Material is added to the wall from vesicles which, in some cells, appear to be derived directly from the Golgi apparatus.⁽²⁾ The rate of Golgi vesicle production, and the time required for a vesicle to traverse the cytoplasm to the cell membrane and wall,⁽³⁾ are compatible with the appearance of visible curvature in geotropism.

What role could the Golgi apparatus play in plant geotropism? It could have the role of a sensor. Griffiths and Audus⁽⁴⁾ found a small but significant displacement of the Golgi upon geostimulation of roots of *Vicia faba*. They considered the displacement to be too small and too inconsistent to form a valid basis for speculation. We will present data that make the Golgi apparatus a possible contender for the role of a geosensor. On the other hand the Golgi apparatus could function in the physiological implementation of geotropism. Geotropic stimulation results in a change of auxin distribution within an organ. The increased auxin concentration on the lower half of a cell could bring about an activation of Golgi apparatus in that part of the cell. The activation of Golgi apparatus by auxin is supported by the thesis work of Siegesmund⁽⁵⁾ with tobacco cells in culture; more Golgi vesicles were formed in those cells when auxin was added to the medium.

Activation of the Golgi is manifest as an increased

production of vesicles by the Golgi.⁽¹⁾ The increased vesicle production could result in an increased rate of auxin transport. Although there is no direct evidence of vesicle effect on auxin transport, unpublished work of Thornton⁽⁶⁾ shows that phototropic stimulation of *Phycomyces* sporangiophore reduces significantly the number of vesicles. We have found that phototropic stimulation of the oat coleoptile inhibits materially the basipolar transport of auxin.⁽⁷⁾ We could infer from this that vesicles might be involved in auxin transport. Further, recent works of Leopold and Lam⁽⁸⁾ and Hertel and Flory⁽⁹⁾ show that pretreatment of tissues with auxin enhances the subsequent transport of auxin. Thus, a feed-back interactive enhancement between Golgi vesicle production and auxin transport does not seem unreasonable. With these considerations in mind, we undertook to examine changes of the Golgi apparatus in oat coleoptiles after various periods of gravity stimulation, using electron microscopy.

After geostimulation the tip 1 mm of the coleoptile tissues was divided into upper and lower halves with respect to gravity and fixed in 2% KMnO₄. The tissues were embedded in Epon resin and sectioned. All tissues were blind-coded to minimize subjective bias. In preliminary scanning, we scored the number of Golgi bodies and their activity in the top and bottom halves of cells in the upper and lower region of a coleoptile segment.

PROGRESS REPORT

We have arbitrarily segregated the Golgi apparatus into the following three states: the inactive (N), those of intermediate activity (I), and the active (A) (Figure 268). It is obvious that the N are smaller in

FIG. 268.—Three types of dictyosomes of the oat coleoptile tip: N, inactive; I, those of intermediate activity; and A, active.

FIG. 269.—The effect of gravity stimulation on the distribution of the dictyosomes in the upper and lower tissues (with respect to gravity) of coleoptile tips (1 mm) of oats ($n = 25$ cells). The diagram represents a cross-section of a coleoptile tip: N, inactive; I, those of intermediate activity; and A, the active.

size both in thickness and diameter of the cisternae: 0.78μ (N) in diameter as compared to 1.3μ (I). The activity of the Golgi is determined by their vesicle production. There is almost no production in the N, some in the I, and much in the A.

Figure 269 shows the distribution of total dictyosomes per cell section in the coleoptile tip. In the upper tissue there were always more N dictyosomes at the various stimulation times. The I increases and then decreases, the A increases slowly with increasing stimulation. The distribution of dictyosomes in the lower tissue is different from that in the upper. The N decreases steadily at longer stimulation times, and the A shows a steady increase. Table

TABLE 120.—TOTAL NUMBER OF DICTYOSOMES PER CELL SECTION IN THE UPPER AND LOWER HALF OF OAT COLEOPTILE TIP TISSUE, GRAVITY STIMULATED BY VARIOUS LENGTHS OF TIME

Stimulation time, min	Total No. of dictyosomes	
	Upper tissue	Lower tissue
0	8.0	8.0
30	9.1	9.7
60	10.0	8.6
90	9.4	10.0

(*) Each datum represents an average of 25 cells.

120 indicates essentially no difference in the total number of dictyosomes per cell section between the upper and lower tissues at the various stimulation times. It must be pointed out that we did not have comparable vertical controls for the different stimulation times. Thus it is possible that nongeostimulated cells also increase in dictyosome number with time.

Figure 270 shows the distribution of dictyosomes between the top and bottom half of a cell. The bottom half always has more than the top, both in the upper and lower tissues. The increase in the bottom could be a result of production of new dictyosomes or a transfer of dictyosomes from the top to the bottom, or perhaps both. The question of transfer or production has to be settled more rigorously before the Golgi apparatus can be seriously proposed as a geosensor. Figure 271 shows the distribution of active dictyosomes between the top and bottom half-cells. The bottom has more active dictyosomes than the top. This difference is more striking in the lower tissues than in the upper.

FIG. 270.—The effect of gravity stimulation on the distribution of dictyosomes in the top and bottom half-cells in the upper and lower tissues of oat coleoptile tips ($n = 25$ cells).

FIG. 271.—The effect of gravity stimulation on the distribution of active dictyosomes in the top and bottom half-cells in the upper and lower tissues of the oat coleoptile tip ($n = 25$ cells).

CONCLUSION

In geotropic curvature, the cells in the lower tissues elongate more than those in the upper. Our data show that the bottom half of a cell has both more dictyosomes and more active dictyosomes than the top

after geostimulation. The difference in the number of active dictyosomes is greater in the lower tissue than in the cells of upper. These preliminary observations suggest that activity and the localization of activity of the Golgi apparatus are correlated with the direction of the gravitational field, and that the morphological changes in this organelle occur with time constants not incompatible with the appearance of geotropism.

Since the latent period between gravity stimulation and the appearance of visible curvature is relatively short, ca. 15 min, we are obtaining data for stimulation periods between zero and 30 min, to check if both Golgi distribution and activation can be correlated kinetically with the georesponse. Since geotropism is a consequence of altered auxin distribution, we plan to determine 1) if auxin affects vesicle production and 2) if the rate of auxin transport is related to the number of vesicles. If both phenomena occur, their sequence in geotropism still remains to be determined. Both processes could well be feedback related.

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CYTOLOGY OF THE YOUNG OAT SEEDLING

Ray Hinchman

PURPOSE AND METHODS

Though the oat seedling (*Avena sativa*) has been used extensively in plant physiological research, there is relatively little detailed cytological information about the early stages of its germination. Our preliminary observations indicate that very young oat coleoptiles (less than 2 mm long) do not respond to geotropic stimuli. Because of the possible role of the coleoptile amyloplast in geotropic response, we have begun an investigation of the starch economy of the young oat seedling. More specifically, we are determining patterns of starch mobilization from endosperm reserves to coleoptile amyloplasts, examining in particular interactions at the endosperm-embryo interface.

This report describes a number of cytological observations on the early stages of germination in *Avena*, and focuses on the location, morphology and evidence of mobilization of starch.

Seeds of *Avena sativa* cv. Victory, were soaked in tap water at room temperature for 2½ hr, drained, and placed at 2°C for 18 hr. At the end of the cold treatment, some of the seeds were fixed in warm FPA (formalin, propionic acid, alcohol) under vacuum (0-hour samples). The remainder were placed on moist filter paper in the dark at 25°C. After 24 hr, seeds that had germinated were selected under dim green safe-light. Some of these germinants were fixed as above (24-hr samples). The remainder were planted in moist quartz sand and allowed to grow for 48 hr, at which time they were also fixed (72-hr samples). Fixed specimens were dehydrated, embedded in Tissuemat, sectioned, and stained with a quadruple stain (Mayer's acid haemalum, safranin, fast green and orange G), acid fuchsin, or IKI.

PROGRESS REPORT

The two major parts of the oat seed, or caryopsis, are the endosperm and the embryo (Figure 272a). At zero hour, there is a slight increase in size of the embryo, primarily due to imbibitional swelling. At 24 hr, the shoot is less than 2 mm long and the single primary root can be up to 5 mm in length. At 72 hr, the shoot is 10 to 15 mm long and the 4 or 5 roots vary from 2 to 30 mm in length.

The aleurone layer is the outermost tissue of the endosperm, and in oats is 1 to 2 cells thick (Figures 272b, c and Figure 273a, b). A number of physiological studies have shown that barley aleurone cells

secrete α -amylase in response to gibberellic acid (GA) secreted by the scutellum.⁽¹⁾ The α -amylase breaks down the starch of the endosperm and converts it to soluble carbohydrates that are absorbed by the scutellum.

The aleurone cells are densely cytoplasmic and have thick cell walls. The cells are packed with aleurone grains, a characteristic feature. In the aleurone cells of oats, a gradual increase in the size of the aleurone grains with time was found, with the maximum size reached in the 72-hr samples (Figures 273a and b). A marked erosion and lysis of the walls of the aleurone cells of the 72-hr sample was observed. Paleg⁽²⁾ reported similar changes in isolated barley aleurone treated with GA.

In oats, only a portion of the aleurone layer appears to be active (as indicated by increased aleurone grain size) at a given time during germination. The aleurone cells nearest the scutellum are activated first, and activation proceeds from the embryo end of the grain to the chalazal end. By the time the aleurone cells at the chalazal end of the seed are activated, many of those nearest the scutellum are lysed and empty.

The endosperm of the mature, ungerminated oat seed is completely cellular and the major storage product is starch. After imbibition (0 hour), the endosperm is still cellular. In Figure 272b, several endosperm cells which contain starch plastids (amyloplasts) are visible. These contain from one starch grain to several hundred polyhedral granula as a compound grain.

At 24 hr, extensive breakdown of the endosperm cells has taken place. Most of the starch is in the form of free granula and there is no evidence of cell walls, cytoplasm or amyloplast membranes (Figure 272c). At 72 hr the endosperm starch is greatly depleted and the granula that remain are noticeably smaller due to enzymatic erosion (Figure 272d). Even at this stage, there may be a few intact endosperm cells at the chalazal end of the seed, indicating the progressive nature of the endosperm dissolution.

The scutellum is an elongate shield-shaped structure that is part of the embryo, has a well-developed vascular bundle, and is in contact with the starchy endosperm (Figure 272a). All endosperm metabolites translocated to the embryo axis must move through the scutellar interface. Thus, it appears the scutellum has a dual function: the secretion of GA

FIG. 272.—Changes in oat scutellum during germination: a, embryo end of oat seed showing embryo and part of endosperm ($\times 44$); b, oat scutellum, 0 hr; c, oat scutellum, 24 hr; d, oat scutellum, 72 hr (b, c, d, $\times 158$). A, aleurone layer; AR, adventitious root; CP, coleoptile; CR, coleorhiza; E, epiblast; L, leaf (first); R, radicle; S, endosperm starch grain(s) or granula; SA, shoot apex; SC, scutellum (cortex); SCE, scutellum epithelium; SE, cellular starchy endosperm tissue; V, vascular bundle of scutellum.

FIG. 273.—Changes in oat aleurone cells and scutellum amyloplasts during germination: a, Aleurone layer at 0 hr; note small aleurone grains and cellular endosperm. b, Aleurone layer at 72 hr; note large aleurone grains and free starch (a, b $\times 253$). c, Scutellum cortex and epithelial layer (24 hr) stained with IKI; note numerous small amyloplasts and large grains and granula of free endosperm starch. d, Scutellum cortex and epithelial layer (72 hr) stained with IKI; note large amyloplasts (c, d $\times 400$). A, aleurone layer; AG, aleurone grains; AP, amyloplasts; S, endosperm starch grains; SC, scutellum (cortex); SCE, scutellum epithelium; SE, cellular starchy endosperm.

FIG. 274.—Changes in oat scutellum epithelium during germination: a, portion of scutellum (0 hr) showing unexpanded epithelial layer ($\times 158$); b, higher magnification of same area ($\times 400$); c, portion of scutellum (72 hr) showing expanded, villi-like epithelium ($\times 158$); d, higher magnification of same area ($\times 400$). SC, scutellum (cortex); SCE, scutellum epithelium; SE, cellular starch endosperm.

and absorption of the products of starch hydrolysis. To accomplish these functions effectively, the scutellum undergoes considerable expansion and develops villi-like cells from the epithelial layer.

At 0 hour, the cells of the scutellum are compact and densely cytoplasmic; the epithelial layer is unexpanded (Figures 272b and 274a and b). At 24 hr, the scutellum has expanded to more than double its size at 0 hour and extends half the length of the endosperm area. The cortical cells have become vacuolate and contain many small amyloplasts and nonstarch inclusions (Figures 272c, 273c). The cells of the epithelial layer are expanding and becoming long and cylindrical. At 72 hr, the scutellum cortex cells are very long and appear to be degenerating. Those near the base of the scutellum contain many large amyloplasts (Figure 273d). The epithelial cells are even more expanded and extended at this time (Figure 272d and 274c and d).

At 0 hour, no starch is found in the embryo stained with IKI. At 24 hr, there are numerous amyloplasts throughout the scutellum and embryo axis. The starch that appears in the embryo after germination is markedly different, in form and appearance, from the endosperm starch. The endosperm starch, at all sampling times, has a much darker IKI reaction. The endosperm starch, prior to germination, is contained in amyloplasts in the form of very large compound grains. After germination, this starch is found free in the endosperm space as polyhedral granula, which become progressively smaller, probably by enzymatic erosion.

The starch grains in the cells of the embryo are smaller and usually occur in numbers of eight or less per amyloplast. The grains in the larger amyloplasts are often eumate in shape, arranged around a center with areas of nonstaining matrix between the grains (Figure 273d, arrow). Numerous starch grains were observed, even in the epithelial cells of the scutellum at 24 hr, but no starch was found in the epithelial cells at 72 hr (Figure 273c and d). It may be suggested that, during germination, the starch of endosperm origin is mobilized and deposited several times before it is utilized in the embryo axis.

CONCLUSION

The cellular endosperm of the oat seed breaks down soon after germination, and its reserve starch is hydrolyzed and translocated rapidly to amyloplasts throughout the embryo. The scutellum and embryo axis contain no starch or amyloplasts at the time of germination. The lack of geotropic sensitivity early in germination possibly is correlated with the absence of this polysaccharide organelle. During germination, the scutellum expands and develops a villi-like epithelial layer. The development of geotropic sensitivity of the very young oat coleoptile will be examined in subsequent studies.

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INFLUENCE OF AUDIOFREQUENCY SOUND ON THE GROWTH OF THE OAT SHOOT: FURTHER OBSERVATIONS

Richard A. McPherson* and Solon A. Gordon

PURPOSE AND METHODS

Several plant species flown in Soviet and American space flights were found to have more chromosome or cytological aberrations than their ground-based counterparts. These differences have been attributed to unique vibrational stresses. It is possible that organisms in free fall will respond to vibrational acceleration in fashions qualitatively and quantitatively different from those under terrestrial conditions. It is of interest, therefore, to determine how gravity-compensated plants respond morphologically and cytologically to vibration.

* Student Aide.

As a first step, we examined⁽¹⁾ the effect of acoustic vibration on elongation rates of static oat shoots grown in normal orientation. A reduction in coleoptile growth rate was observed when seedlings were exposed to wide range (50 to 10 kHz) white noise. Moreover, the effects of 500-hertz bands within this range shifted from growth depression to growth acceleration as the frequency rose, with an indication of bimodality at octave separation. Though the differences observed were significant statistically, we were troubled about the validity of the correlations drawn from the data. Resonance excitation of biological structures by audiofrequencies of 1 to 5 kHz suggests dimensions of the order of 100 to 20 cm. For

the oat seedling, resonance of the shoot might be expected at the 5th or 6th harmonic, and of cells in the range of the 13th to 15th harmonic. It seemed improbable that biologically detectable power at these harmonic levels was generated by the equipment used. Accordingly, we have re-examined the effect of both wide- and narrow-band acoustic vibration on the growth of the young oat shoot, paying particular attention to test chamber and temperature effects.

The methods were described previously.⁽¹⁾ Essentially, they involve growing oat seedlings, whose mesocotyl had been inhibited by brief exposure to red light, in the dark for three days. During the last 19 hr of this period, they were exposed to white noise in "anechoic" chambers constructed of Styrofoam. The influence of the experimental treatments on growth rate was determined by measurement of organ increments in length on shadowgraphs taken at the beginning and end of the experimental treatment. Phototropically inactive green light was used for photography.

PROGRESS REPORT

Seedlings were exposed to wide- and narrow-band white noise, at about 40 to 45 db above background level. The experiments were designed on Latin square matrices to permit evaluation of the influence of location effects, specifically, the influence on growth rate of the Styrofoam chamber itself and the influence of small differences in temperature. With this experimental design, we find no significant retardation or enhancement of growth of the coleoptile under the influence of either wide-band acoustic noise in the frequency range of 50 to 10,000 hertz, or narrow bands (-4 db at 20% each side of midfrequency, dropping 24 db per octave) at 500, 1,000, 2,000, or 4,000 hertz.

Significant enhancements in the growth rate were obtained when the seedlings were exposed to wide-range noise at 70 db above background. However, the acoustic driver became quite warm in the generation of these power levels. Tests of the temperature in the experimental chambers in the 70 db field (130 db against reference sound pressure of 2×10^{-4} dynes/cm²) showed a gradual rise in air temperature over a 7-hr period, stabilizing at 1.4°C above the room temperature of 23.3°C. We know that the rate of coleoptile extension increases with temperature in this temperature range, although the exact relationship has not been determined. Thus, it seems likely that the increase in growth (6.5%) observed

at the 130-db sound levels occurred, wholly, or in part, as a consequence of the rise in temperature.

Assuming, for the ranges of temperatures, T , and velocity of growth, v , encountered, that $v = K T$, it can be shown that the coefficient of velocity increase

$$K = F \left[\frac{v_1 t_1 + v_2 t_2}{t_1 \Delta T_1 + t_2 \Delta T_2} \right], \quad (1)$$

where F is the fractional increase of growth occasioned by a temperature increase during a period wherein the growth can be characterized with respect to time, t , by two successive linear rate constants.⁽²⁾ Averaging the areas under the temperature curves that correspond to $v_1 t_1$ and $v_2 t_2$, and using the observed F of 6.5%, the coefficient of velocity increase with temperature was approximated as 0.038 mm·hr⁻¹ °C⁻¹. It would be of interest to test this approximation for the effect of temperature on growth rate of the coleoptile by direct examination of $v = f(T)$.

CONCLUSION

No rigorous inferences may be drawn at this time concerning the effect of white noise, in the range of 50 to 10,000 hertz at sound levels up to 130 db above aural threshold, on the elongation of the *Avena* coleoptile. In the experiments described, cell division in the coleoptile terminated soon after the plants were placed in the acoustic field, and organ elongation then derived solely from cell expansion. We suggest that it may be useful to examine the influence of acoustic vibration on organs in which cell division is continuously involved in the growth process, viz., the oat shoot immediately after germination, the root, or the shoot under a functioning apical meristem. The magnitude of the effect (if any) of sound on coleoptile elongation in the system examined is small with respect to the influence of temperature and chamber components. Under these circumstances, the use of the test organism as its own control might be informative—viz., does introduction of noise in a period of constant growth rate alter the rate constant?

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THE EFFECT OF THE LENGTH OF THE PERIOD OF PREPLANTING IMBIBITION ON SUBSEQUENT GROWTH AND GEORESPONSE OF OAT SEEDLINGS

Louis H. Decker, Edward Rakosnik, Jr., and Richard R. Dedolph

PURPOSE AND METHODS

To obtain more uniform populations of oat seedlings for experimental work, seeds are customarily soaked in warm tap water for about 2 hr prior to planting. It has been reported that holding soaked oat seed under moist conditions overnight in a refrigerator at 5°C "resulted in more uniform germination and growth of seedlings." (1)

To test this, oat seeds (*Avena sativa* cv. Victory 1) were soaked for 2 hr at 25°C and then held under moist conditions at 5°C either for an additional 20 hr (long imbibition treatment) or for only 2.5 hr (short imbibition treatment). The seeds from these two treatments were then planted in moist quartz sand (about 15% water by weight). Seedlings were grown at 25°C for about 69 hr. Red light (G.E. Ruby Red, 1 mW/cm²) was used during the first 50 hr to inhibit the growth of the mesocotyl. Growth was otherwise in darkness, except for brief periods under the same red lights during manipulations necessary for geotropic stimulation.

Shortly before harvest, one-half of each preplanting imbibition treatment class was subjected to a 1.0 × *g* unilateral stimulus for 40 min. After the 40-min stimulation period, plants were rotated on a clinostat (2 rpm) for an additional 40 min to permit curvature development. They were then shadowgraphed to assess coleoptile curvature, harvested, separated as to tops and roots, weighed, and reshadowgraphed for length measurements.

PROGRESS REPORT

No significant (5% level) increase in germination attributable to imbibition period was found with high levels of germination apparent in both the long and

TABLE 122. THE EFFECT OF THE LENGTH OF PREPLANTING IMBIBITION PERIOD ON SUBSEQUENT GROWTH OF CA. 69-HR-OLD OAT SEEDLINGS

Measurement	22-hr period	4.5-hr period
Length of tops, mm	32.9a	28.8b
Length of roots, mm	139.0a	131.5b
Weight of tops, g	0.1472a	0.1322b
Weight of roots, g	0.1688a	0.1603a
n = 60		

Means within rows, not followed by a common letter are significantly different at the 5% level.

TABLE 123. COEFFICIENTS OF VARIATION OF GROWTH AND GEOCURVATURE RESPONSES OF CA. 69-HR-OLD OAT SEEDLINGS IN RELATION TO THE LENGTH OF PREPLANTING IMBIBITION PERIOD

Plant response	22 hr period	4.5-hr period
Curvature, deg	24.8	37.8
Length of tops, mm	3.3	2.9
Length of roots, mm	5.0	4.0
Weight of tops, g	18.0	10.7
Weight of roots, g	21.4	14.0
n = 60		

short imbibition treatments, 96.2 and 98.3%, respectively. The magnitude of geotropic curvature of plants was, similarly, not significantly influenced by the length of the period of preplanting imbibition (Table 121), although the period of stimulation showed the expected differences in eliciting curvature.

The longer period of preplanting imbibition, however, did tend to produce significantly larger plants (Table 122).

These differences in subsequent growth attributable to period of preplanting imbibition are explicable on a basis of a significantly (1% level) greater uptake of water by the seeds permitted to continue imbibition over the longer time period as contrasted to the shorter period (29 vs. 19% of initial weight, respectively). The more fully imbibed seeds likely had progressed further into the initial stages of germination than the less fully imbibed seeds and consequently may have been somewhat older physiologically at the time they were planted.

When coefficients of variation were calculated for the various means, it was apparent that the long preplanting imbibition period resulted in a more uniformly responding population only when the curva-

TABLE 121. THE EFFECT OF THE LENGTH OF PREPLANTING IMBIBITION ON SUBSEQUENT CURVATURE RESPONSES OF CA. 69-HR-OLD OAT SEEDLINGS

Treatment	Curvature, deg	
	22-hr period	4.5-hr period
0.0-min stimulation	2.68a	1.72a
40.0-min stimulation at 1.0 <i>g</i>	29.9b	30.88b
Mean curvature	16.3a	16.3a
n = 30		

Means within rows, not followed by a common letter are significantly different at the 5% level.

ture response was considered. If growth, either as length or weight, was measured, the longer preplanting imbibition period resulted in a less uniform population (Table 123). Tests of homogeneity of variance further showed that the difference in uniformity of the populations of plants grown from seeds that received the two different periods of preplanting imbibition was great enough to render direct comparisons of mean curvatures, weights, or lengths from these two treatments by analysis of variance methods not rigorous.

CONCLUSION

The tenet⁽¹⁾ that a long (20 hr) low temperature (5°C) preplanting imbibition period for oat seeds results in more uniform germination and growth must be rejected on a basis of these data because this treatment resulted in a seedling population less uniform in weight and length than seedlings derived from seeds

that received a short (2.5 hr) treatment period under these conditions. The length of the period of preplanting low temperature imbibition had no effect upon germination.

Seedlings grown from seeds that received the long, low temperature preplanting imbibition, however, were more uniform in their curvature responses to geostimulation and attained greater average lengths and weights than corresponding seedlings grown from seeds that received the shorter treatment period. Consideration of population uniformities suggested that without appropriate transformations, statistical differences between mean lengths, weights, and curvatures could not be rigorously assessed by tests that required homogeneity of error variance.

REFERENCE

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A NOTE ON GROWTH RATES OF THE AVENA COLEOPTILE

Richard A. McPherson* and Solon A. Gordon

PURPOSE AND METHODS

Knowledge of the growth rate of the *Avena* coleoptile during the latter portion of its ontogeny became desirable in a study of acoustic vibration and growth.⁽¹⁾ Accordingly, the elongation rate of the etiolated coleoptile was determined by hourly measurement of height increments for a 24-hr period, beginning two days after seed imbibition.

Seeds were soaked for 2 hr in warm tap water and then stored in the dark at 4°C for ca. 19 hr. They were then aligned on moist sharkskin filter paper on Lucite bars and exposed to red light for 24 hr at 25°C. Each seed was planted individually in a metal cup and grown in the dark at 26.7° for 27 hr in one experiment, 31 hr in another, corresponding to 51 and 55 hr of growth after the imbibition at low temperature. At this point, photography of the heights of the coleoptiles was initiated and continued at hourly intervals. Green light, λ max. 560 nm, 1% width 40 nm, phototropically inactive at the exposure levels employed, was used for photography. Growth was computed by measuring incremental differences in height as a function of time. Parallel runs of seedlings were photographed only at the beginning and end of the 24-hr growth period, or at either 6- or 10-hr intervals.

* Student Aide

The height increments were slightly less for the plants photographed hourly. However, the relation between growth increments and amount of photographic manipulation was not monotonic, and the rate changes in the plants photographed at 6-hr intervals were qualitatively similar to those photographed hourly.

FIG. 275. - Height increments of the intact etiolated *Avena* coleoptile during the third day of growth. I and II are the growth curves of separate experiments. Initial rates and intercepts are represented, respectively, by v_1 and a_1 ; v_2 and a_2 are the constants for the later portions of the curves.

PROGRESS REPORT

The increments of growth measured over the third day of coleoptile elongation are shown in Figure 275. Within each experiment, regressions were computed for the two growth phases (breaking at 11 hr) separately and then for the entire time period (51 or 55 hr to termination). In each instance, two constant rates fit the data better than a single quadratic curve at a chance probability of less than 0.5%. The velocity constant for the first growth phase is about $0.5 \text{ mm} \cdot \text{hr}^{-1}$, for the second about $0.9 \text{ mm} \cdot \text{hr}^{-1}$.

We find these curves, describing the growth rate of the intact coleoptile, of interest in two respects. First, they are qualitatively dissimilar to published data on the growth rates of *Avena* coleoptiles at different ages;⁽²⁾ the latter show an almost constant increase of growth rate from about the 15th to the 65th hr after soaking; the rate decreases progressively after 70 hr.

Second, it is known that cell divisions accompany elongation of the parenchyma cells up to a coleoptile length of about 1 cm; thereafter, growth is entirely by cell elongation.⁽³⁾ The length of the organ at this developmental change corresponds to the length of the coleoptiles at the intersection of the two growth curves (Figure 275). It may be suggested that the two processes, cell division and elongation, compete for a factor limiting to organ extension.

This factor could well be gibberellin. There is evi-

dence that, in barley, the scutellum is the site of gibberellic acid (GA) origin in the early stages of germination.⁽⁴⁾ The production of GA by the scutellum terminates near the third day of germination; the seedling axis then takes over with the, apparently *de novo*, synthesis of GA.^(4, 5) It is likely that a similar phenomenon occurs in the oat, and it would be of interest to determine whether the change in growth rate we observe could be correlated with this transition of GA production. Possibly also related to these GA and growth phenomena is the observation that at approximately transition time, amyloplast number and size rise markedly in the scutellum of the oat.⁽⁶⁾

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EDUCATIONAL ACTIVITIES IN COOPERATION WITH THE ARGONNE CENTER FOR EDUCATIONAL AFFAIRS

SUMMER INSTITUTE IN RADIOBIOLOGY, JUNE 16–AUGUST 1, 1969

As a part of a national effort to further the education and training of science teachers, Argonne National Laboratory offered a seven-week program of instruction for college teachers of biology. Prospective participants were informed about the nature of the program through the distribution of a directory of summer programs by the National Science Foundation and a brochure sent out by Argonne's Center for Educational Affairs (CEA).

The institute was a cooperative effort by several participating organizations. As in previous years the initial proposal was sponsored by the Associated Colleges of the Chicago Area (ACCA)* and the lecture and laboratory components of the program were offered by Argonne staff members in several divisions. The Division of Biology and Medicine (BIM) provided lecture hall facilities and the majority of lectures, whereas the CEA used their instructional laboratories for individual and group instruction in laboratory techniques and the operation of instruments.

The program was organized and coordinated by

* Aurora, Concordia, Elmhurst, George Williams, Judson, Lake Forest, Lewis, Mundelein, North Central, North Park, Olivet Nazarene, Rosary, St. Dominic, St. Francis, St. Procopius, St. Xavier, Trinity Christian, and Wheaton.

Raymond H. Brand† who served as Associate Director of the Summer Institute. Major emphasis in the series of lectures was given to basic aspects of radiation and the effects of radiation on biological systems. Additional topics in molecular biology were also included. The sequence of topics ranged from molecular and cellular biology to the organism, population, and ecosystem levels of organization.

Twenty-one college teachers took part in the program. Of these, six were from two-year colleges and fifteen from four-year colleges or universities. Participants and their respective institutions and geographic locations are indicated in the accompanying photograph.

One of the major objectives of this particular institute program is to bring biology teachers from academic environments where teaching has primary emphasis to an institution where research is of primary importance. It is believed that through the experience of contact with persons engaged in active scientific research the teacher will be better informed about recent developments in biology. In addition, he may be stimulated to initiate or continue a modest research program on his own campus and be in a better position to guide students in limited research projects.

† Professor of Biology and Chairman of the Science Division at Wheaton College, Wheaton, Illinois.

Fig. 276

<i>Individual</i>	<i>Institution</i>	<i>Location</i>
<i>Front Row</i>		
Jerome Burd	Argonne National Laboratory	Argonne Ill
Orty Orr	Missouri So. College	Joplin Mo
Mary Lou Lechowich	Argonne National Laboratory	Argonne Ill
Mustafa Sayed	Fort Hays Kansas St. College	Hays Kans
Alex Barthel	North Park College	Chicago Ill
Ruth Helstrom	Muskegon Community College	Muskegon Mich
Mary Nemmer	Alverno College	Milwaukee Wis
Joseph Otiero	University of Michigan (Ann Arbor)	Ann Arbor Mich
Beatrice Knoeller	Springfield College	Springfield Ill
<i>Middle Row</i>		
Raymond Brand	Wheaton College	Wheaton Ill
Noble Roberts	Campbellsville College	Campbellsville Ky
Henry Kriss	College of DuPage	Naperville Ill
Chester Vander Zee	Sioux Empire College	Howardsville Iowa
Elmer Jos. Hendricks	San Jose State College	San Jose Calif
Kent Martin	Otero Jr. College	La Junta Colo
<i>Back Row</i>		
Louis Tenhund	Washburn University	Topeka Kans
Clinton Muck	Wheaton College	Wheaton Ill
William Willers	Wis. St. University	Oshkosh Wis
Arthur Wagner	Joliet Jr. College	Joliet Ill
Dale Taylor	Millikin University	Decatur Ill
Glenn Peterjohn	Baldwin Wallace College	Berea Ohio
Gail Norris	Denison University	Granville Ohio

SYMPOSIUM ON THE EVOLUTION OF THE IMMUNE RESPONSE— OCTOBER 20 TO 22, 1969

Bernard N. Jaroslow, Chairman

The 4th annual AUA-ANL Biology Symposium examined the evolution of the immune response. These symposia are aimed at graduate students in the AUA member universities. Symposium topics are selected from subjects identified with promise for future growth where new investigators can find many stimulating problems. Evolution of vertebrate immunity was discussed in depth at molecular, cellular, and organismic levels. The present sketchy knowledge of invertebrate immunity was examined in comparison with that of vertebrates. Three hundred eighty registrants attended the symposium. Sixty percent of these were graduate students from AUA member universities. The remainder included staff and students from ANL and staff from AUA member universities.

THE PROGRAM

Monday, October 20

Welcoming Address

R. B. Duffield, Director, Argonne National Laboratory

Introduction and Announcements

B. Jaroslow, Argonne National Laboratory

I. REGULATION OF GAMMA GLOBULIN SYNTHESIS

R. E. Ecker, Chairman, Argonne National Laboratory

Genetics of Antibodies

O. Smithies, The University of Wisconsin

Quantitative Studies of Idiotype Antibodies

A. Nisonoff, University of Illinois, Medical School

II. EVOLUTION OF GAMMA GLOBULINS

J. J. Cebra, Chairman, The Johns Hopkins University

Antibody Complementarity and Light Chain Structure

E. A. Kabat, Columbia University

The Genetic Basis for Antibody Diversity

L. Hood, National Institutes of Health

Physical and Chemical Properties of the Immunoglobulins

A. B. Edmundson, Argonne National Laboratory

Tuesday, October 21

III. EVOLUTION OF VERTEBRATE IMMUNITY

D. Talmage, Chairman, University of Colorado

Phylogenetic Development of Lymphoid System, Immunoglobulins and Immune Responses

R. A. Good, University of Minnesota

Evolution of Vertebrate Immunoglobulins

B. Papermaster, Roswell Park Memorial Institute

Evolution of the Complement System

H. Gewurz, University of Minnesota

IV. TRANSPLANTATION IMMUNITY

R. T. Smith, Chairman, University of Florida

Evolution of Immune Responsiveness in Ectothermic Vertebrates

W. H. Hildemann, University of California

Serological and Cellular Aspects of Immunity in Several Marine Invertebrates

F. B. Bang, The Johns Hopkins University

Correlations of Radiation Sensitivity with Immunologic Responsiveness in Phylogeny

J. Finstad, University of Minnesota

INTERDISCIPLINARY LECTURE

J. Thomson, Chairman, Argonne National Laboratory

Biological Benefits of Technology

D. W. Bronk, The Rockefeller University

Wednesday, October 22

V. NON-VERTEBRATE IMMUNITY

J. Cushing, Chairman, University of California

Cell vs. Cell in the Cockroach

C. J. Dawe, National Institutes of Health

Humoral Immunity in Insects

J. Stephens Chadwick, Queen's University

Disease Immunity in Plants

J. Paxton, University of Illinois

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