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ARGONNE NATIONAL LABORATORY

BIOLOGICAL AND MEDICAL RESEARCH DIVISION

ANNUAL REPORT

1965

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Dosimetry

JANUS REACTOR. I. PRELIMINARY DOSIMETRY IN THE EXPOSURE ROOMS

Frank S. Williamson, Donn L. Jordan, and Richard D. Hudnut

PURPOSE AND METHODS

Measurements made at very low power levels have been reported previously.⁽¹⁾ It was evident from these data that the design ratio of 10,000/1 in neutron dose rate between the high-level and low-level faces of the reactor would not be achieved. It seemed very likely that at 200 kW the dose rate in the low-level room would be approximately as expected, but that the dose rate in the high-level room would be low by a factor of about ten. Measurements of flux distribution along the thermal column from the core to the high-level face were carried out by the Reactor Operations Division and supported this prognostication.⁽²⁾

It was decided, therefore, to install a graphite-filled tank which would displace most of the water moderator between the core and the thermal column. This modification was expected to produce an improvement, of a factor between 3 and 4, in neutron flux at the high-level face. At the same time a voidable water tank (the Low-Level Flux Adjuster) was fitted in the moderator, on the low-level side, to provide an optional increase in the neutron flux at the low-level face.

The first power calibration of the reactor took place on June 11, 1965 at 15.6 kW, and from June 30 the reactor became available for dosimetry studies at the full power of 200 kW.

Preliminary measurements of fast-neutron and gamma dose rates have been made using a pair of ionization chambers. One chamber consists of a plastic wall having the same atomic composition as the acetylene gas filling, and is sensitive to both neutrons and gamma rays. The other chamber has a magnesium wall and argon gas filling, and responds mainly to gamma rays.⁽³⁾ Currents from these chambers were measured using a vibrating reed electrometer (Cary Model 31) to determine the potential drop produced across a high value resistor (10^8 , 10^{10} , or 10^{12} ohms). In plotting the variations of dose rate against an additional parameter, it was necessary to eliminate the effect of fluctuations in reactor power. This was done very conveniently by

setting up a second electrometer channel to read the current from a tissue-equivalent ionization chamber,* which acted as a monitor. The output of the monitor channel was applied to the X axis, and the output of the measuring channel to the Y axis, of a Moseley X-Y recorder. Any variation of reactor power extended the single point recorded into a straight line passing through the chart origin. The slope of this line, Y/X , gave the ratio of the currents from the measuring and the monitoring ionization chambers.

PROGRESS REPORT

Measurements of neutron dose rate at each face, 1 meter from the lead face, along the central-room axis at mid-height of the face, yielded a high-level/low-level ratio of 3,770, with the Low-Level Flux Adjuster filled, a factor of improvement close to that expected from the modifications. In Table 1 are summarized the neutron and gamma dose rates in both exposure rooms for various conditions of exposure.

High-Level Exposure Room

Figure 1 shows the neutron dose rate, gamma dose rate, and gamma/neutron dose ratio (%) at points along the center line of room at the mid-height of the face.

There is a lead brick wall, 6 inches thick, between the converter (fission) plate and the High-Level Room. In order to determine whether or not this wall is of optimum thickness for the reduction of gamma-ray contamination, measurements were made with the center section removed. The ionization chambers were set up at 1 meter from the face along the central room axis, at the mid-height of the face, and surrounded on 5 sides of a cube with 2 inches of lead to shield against gamma radiation from the interaction of neutrons in the walls of the room. The sixth side, facing the reactor, was filled in with various thicknesses of lead, and the neu-

* Constructed and supplied by Dr. Francis R. Shonka, St. Procopius College, Lisle, Illinois.

TABLE 1. NEUTRON AND GAMMA DOSE RATES IN THE JANUS EXPOSURE ROOMS AT 200 kW REACTOR POWER, WITH THE FISSION CONVERTER PLATES IN USE

Points of Measurement at Indicated Distance from the Reactor Face along the Room Central Axis at the Mid-Height of the Face. Low-Level Flux Adjuster Condition Shown

Location	Low-Level Flux Adjuster condition	Dose rate, rads/hr		
		Neutron	Gamma	Gamma/neutron, %
High-level room 1 meter	Not applicable	3,150	216	6.9
Low-level room 1 meter 5 meters	Filled	0.855	0.22	25.6
		0.127	0.051	40.0
Low-level room 1 meter 5 meters	Voided	7.73	0.88	11.4
		1.18	0.25	21.4

FIG. 1.—Janus High-Level Exposure Room. Distribution of fast-neutron and gamma dose rates, and the gamma/neutron dose-rate ratio along the central axis of the room at the mid-height of the exposure face under normal exposure conditions, i.e., reactor power 200 kW, with fission converter plate.

tron and gamma dose rates were measured. It is not possible to satisfy the conditions for measuring a half-value layer for the gamma rays in lead, but the results, shown in Figure 2, indicate that certainly the last 2 inches of lead thickness (from 4–6 inches) are disad-

vantageous, while 2 inches alone may be quite adequate.

A similar technique was used to examine the gamma radiation produced in the walls of the room. In this case, the lead-walled cube was set up with the face of variable thickness adjacent to the rear wall. The lead brick wall at the reactor face was replaced to minimize gamma radiation from that source. In Figure 3, the

FIG. 2.—Janus High-Level Exposure Room. The effect on fast neutron and gamma dose rates, and the gamma/neutron dose-rate ratio of various thicknesses of lead filtration under normal exposure conditions.

FIG. 3.—Janus High-Level Exposure Room. The effect of various thicknesses of lead shielding on prompt gamma radiation from the rear wall under normal exposure conditions.

ratio (%) of gamma dose rate/neutron dose rate shows clearly that even $\frac{1}{2}$ inch of lead could greatly reduce the gamma contribution due to neutron interactions in the concrete.

Low-Level Exposure Room

Similar measurements have been made in the Low-Level Room. Figure 4 shows the distribution along the central axis of the room, at mid-height of the face, of neutron dose rate, gamma dose rate and gamma/neutron dose ratio (%). The gamma contribution is 15% at the reactor face and rises to a maximum of more than 40% near the rear wall. The shape of the gamma dose rate curve suggests that this radiation is coming from the reactor, through the 4-inch-thick lead wall.

A series of measurements was made at the 1-meter distance using the 2-inch-thick lead-walled cube exactly as it was used in the High-Level Room. The lead brick wall across the reactor face was left intact so that the variable-thickness face of the cube was additional. The results are shown in Figure 5 from which it appears that a further $1\frac{1}{2}$ inch of lead produces a great improvement, but that an even greater additional thickness may be necessary.

The difference in neutron flux at the high- and low-level faces of the reactor is primarily due to the greater attenuation of neutrons from the core, on the way to the low-level face, in passing through the excess of water moderator. In consequence, there will be a great deal of gamma-ray production in this water volume from $H(n,\gamma)$ interactions. There is likely to be more reduction of neutron flux at the low-level face, compared to the high-level face, than there is reduction of gamma rays.

The effect of voiding the Low-Level Flux Adjuster

FIG. 4.—Janus Low-Level Exposure Room. Distribution of fast-neutron and gamma dose rates, and the gamma/neutron dose-rate ratio along the central axis of the room at the mid-height of the exposure face under normal exposure conditions.

FIG. 5.—Janus Low-Level Exposure Room. The effect on fast-neutron and gamma dose rates, and the gamma/neutron dose rate ratio of various additional thicknesses of lead filtration under normal exposure conditions.

tank was determined at 1 meter and 5 meters from the reactor face along the central axis of the room at mid-height of the face.

The ratio of neutron dose rate Flux Adjuster void/Flux Adjuster filled is 9.04 at 1 meter and 9.29 at 5 meters. For the same conditions, the ratio of gamma dose rate void/filled is 4.0 at the 1-meter location. Voiding of the Flux Adjuster reduces the ratio (%) gamma/neutron dose rate from 25.6% to 11.4%. This observation confirms that the high gamma dose-rate component in the Low-Level Room originates in the reactor.

There is no detectable effect on neutron or gamma dose rate in the Low-Level Room, of raising and lowering the converter plate or shutters of the high-level face, for any condition of the Low-Level Flux Adjuster.

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JANUS REACTOR II. PRELIMINARY DOSIMETRY IN THE EXPOSURE ROOMS IN THE SHIELDED CONDITION

Frank S. Williamson and Martin C. Schumacher*

PURPOSE AND METHODS

In the Janus reactor, shutters may be dropped into place and the fission converter plates withdrawn at each exposure face. This design feature is provided in order that an experiment in one exposure room can be set up, or the irradiated subjects recovered, without exposing personnel to radiation hazard. The alternative would be to scram the reactor at the end of an exposure, which would interfere with any experiment in progress in the other exposure room.

The efficacy of this shutter arrangement has been checked by making measurements of the neutron and gamma dose rates in the exposure rooms with the shutters closed, under which condition the electrical interlocks prohibit use of the converter plates.

It is inevitable that when almost any structural (e.g., walls) or shielding material is exposed to neutrons, activation of the material takes place. Measurements have been carried out to determine the gamma dose rate in the rooms arising from neutron activation.

PROGRESS REPORT

Radiation Levels in the Exposure Rooms with the Reactor Operating.

High-Level Room.—Measurements of leakage neutron flux in the High-Level Room with the shutters closed have been made at various values of reactor power from about 0.2 watts to 3 kilowatts using bare BF_3 counters, cadmium-shielded BF_3 counters, and a long counter. A linear relationship with reactor power was obtained. Dose-rate measurements were made at 1.5 and 15 kilowatts using two devices having a response weighted to give rem dose as defined by the ICRP. These were a Texas Nuclear Neutron Dosimeter and a moderating sphere fitted with activation detectors.⁽¹⁾

The results are shown in Table 2 for the two power levels used and also extrapolated to 200 kW.

Reasonable agreement was found between results obtained by the two methods. These data suggest, for the practical situation of an operative making repeated entries to the room on every working day, a maximum time in the room of less than 0.5 minute per week.

Measurements with cadmium-shielded BF_3 counters indicated that a considerable fraction of this leakage

flux is above thermal energy. A vertical scan of the neutron flux at 170 cm from the face suggested that the leakage was predominantly over the top of the shutters. Measurements to determine the gamma dose rate under the same conditions are being carried out.

The first indication is that the gamma dose rate will be approximately equal to the neutron dose rate when expressed in rem.

A remedy for this excessive neutron leakage is being sought. Comprehensive flux and spectrum measurements are being made by N. A. Frigero, and a shielding study, based on these and other data, is being carried out by A. E. McCarthy of the Reactor Physics Division.

Low-Level Room.—Leakage dose rates at 200 kW were directly measured in the Low-Level Room under the following conditions: shutters closed and converter plates out, flux adjuster tank filled. Points of measurement were located on the room central axis at the mid-height of the face at 13 cm, 1 m and 3 m from the reactor face. The results are shown in Table 3 for

TABLE 2. NEUTRON DOSE RATES IN THE JANUS HIGH-LEVEL ROOM IN THE SHIELDED CONDITION

Instrument	Distance from reactor face, cm	Dose rate, rem/hr Power, kW		
		1.5	15	200
Texas Nuclear	13	0.25	—	33
" "	170	0.10	0.98	13
Moderator + foils	170	—	0.89	12

TABLE 3. NEUTRON DOSE RATES IN THE JANUS LOW-LEVEL ROOM IN THE SHIELDED CONDITION

High-level shutter	High-level converter	Dose rate (mrem/hr) at distance			
		13 cm	1 m	3 m	
Closed	Out	35	25	7	
Open	Out	20	—	4	
Open	In	75		15	(Normal irradiation condition)
Closed	In	250		50	(Transient condition)

* Industrial Hygiene and Safety Division.

various conditions of the shutters and converter plate at the other face.

As in the High-Level Room, there is neutron leakage over the closed shutters. The neutron dose rate at the top of the face is about twice that at the center of the face. Whereas no coupling of the two exposure faces was observed in the normal irradiation condition,⁽²⁾ such a coupling was observed at the low-level face with the shutters closed for various conditions of the high-level face, probably due to neutrons scattering without moderation from one face over the top of the shutter at the other face.

Using these data as a basis for estimation of the permissible room occupancy as already determined for the High-Level Room, a maximum time of 4 hours per week is arrived at.

Wall Activation and Gamma Radiation

High-Level Room.—The room was exposed to the neutron flux from the converter plate with the reactor at 200 kW power. After exposures of ½ hour, 2 hours, and 6 hours, the gamma dose rate in the room at mid-height of the face, on the central axis of the room 1 meter from the face, was recorded for periods of up to 19 hours. The results are shown in Figure 6. Attempts at decay-constant analysis of the data are inconclusive after the large contribution from aluminum activation (half-life 2 minutes) has been subtracted.

Spot measurements with a hand monitor in 9 locations fit these curves reasonably well, and confirm that the large bulk of concrete in the walls, floor, and ceiling is responsible.

Since the aluminum activation dies out in a few minutes, it will only be necessary to delay entry to the room in order to avoid this source of hazard. In the case of the concrete, it will clearly be necessary to control the hazard directly. Three possible solutions are currently under review:

- 1) To line the walls with lead.
- 2) To construct additional hydrogenous neutron shielding inside the room so that most of the neutrons are moderated and captured before they can enter the wall.
- 3) To use a neutron-absorbing liner on the walls, which would remove neutrons by capture.

The best solution would be one which would reduce the gamma contribution from the walls during fast-neutron irradiation, and also reduce the flow of degraded neutrons back into the room. An ideal (hypo-

FIG. 6.—Janus High-Level Room. Decay of induced gamma activation after various exposures to fast neutrons from the fission converter plate at 200 kW. Measured at 1 meter from the reactor face on the room central axis, at the mid-height of the face.

thetical) wall would absorb all neutrons incident upon it without scattering and without the production of gamma radiation.

Low-Level Room.—At the present time, wall activation in the room has not been apparent, but a series of 6-hour exposures is planned in order to make measurements of the induced gamma dose rate. It is not expected that activation will present a serious problem, not only because of the much lower neutron flux but because the room is likely to be used for single daily exposures so that frequent entries to the room will not be necessary.

It is a pleasure to acknowledge the contribution of T. A. Steel, who made the measurements with the moderating sphere and activation detectors.

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ABSORBED DOSE DUE TO NEUTRONS OF ENERGIES 0.1 TO 10.0 MEV IN TISSUE OF VARIABLE COMPOSITION

Frank S. Williamson and Paul Mitacek, Jr.

PURPOSE AND METHODS

Some years ago, Frank S. Williamson calculated, from currently-available data, the absorbed dose resulting from fast neutrons of various energies in tissue of variable composition, and graphs derived from these calculations appeared in NBS Handbook 88.⁽¹⁾ Later compilations of the neutron-nucleus elastic-scattering cross sections for the elements of interest, and of the differential cross sections at a greater number of energy intervals, have been published.⁽²⁻⁴⁾ The purpose of this report is to bring these calculations up to date and to outline the future computational program of the authors.

The method used is outlined in Handbook 88.⁽¹⁾ For a neutron of energy E (MeV) and an atom of type i , the mean energy transferred in an elastic collision, E_i , is given by

$$E_i = \frac{2 \cdot A_i \cdot E \cdot \sigma_{i,E} \cdot K_{i,E}}{(1 + A_i)^2} \quad (1)$$

where

$$K_{i,E} = \int_0^\pi (\sigma_{i,\theta,E} / \sigma_{i,E}) (1 - \cos \theta) 2\pi \sin \theta \cdot d\theta$$

$\sigma_{i,E}$ = Total elastic scattering cross section for neutron (barns)

$\sigma_{i,\theta,E}$ = Differential elastic scattering cross section for neutrons scattered at angle θ (barns/steradian)

A_i = Atomic weight of atom of type i .

Hence, the absorbed dose in rads in a pure material of type i , exposed to a neutron fluence of 10^9 n/cm², assuming scattered neutrons can be neglected, is

$$\frac{2A_i \cdot E \cdot \sigma_{i,E} \cdot K_{i,E} \cdot 10^{-24}}{(1 + A_i)^2} \times \frac{6.026 \times 10^{23}}{A_i} \times 1.602 \times 10^{-8} \times 10^9 \quad (2)$$

and consequently

$$\text{Dose (rads) for } 10^9 \text{ n/cm}^2 = 19.31 \times \frac{E \cdot K_{i,E} \cdot \sigma_{i,E}}{(1 + A_i)^2} \quad (3)$$

PROGRESS REPORT

Figures 7-9 give neutron doses in pure carbon, nitrogen, and oxygen, which are markedly different from those in Handbook 88. Figure 10 gives neutron doses in pure hydrogen, which is unchanged from the earlier computation. The dose to a tissue-like material is readily calculated by multiplying the value for each pure constituent by the percentage composition, by

weight, and adding the contributions. For example, soft tissue of ICRP Standard Man composition (H = 10 %, C = 18 %, N = 3 %, O = 65 %, remainder = 4 %) receives a dose of

$$(0.1)(\text{rads for pure H}) + (0.18)(\text{rads for pure C}) + (0.03)(\text{rads for pure N}) + (0.65)(\text{rads for pure O}).$$

The remainder of 4 % heavier atoms makes a negligible contribution to the process of energy transfer. Neutron doses to Standard Man Tissue are also given in Figure 10. Since about 85 % of the dose in Standard Man Tissue is due to neutron interactions with the 10 % by weight of hydrogen it contains, it is apparent that

1) the dose is, to a close approximation, proportional to the hydrogen content of the tissue under consideration;

2) the effects of the resonance peaks in C, N, and O are minimized for broad neutron-energy spectra. Accurate knowledge of the C, N, and O collision processes is essential when monoenergetic neutrons are involved and/or when measurements are made using ionization chambers in which plastics and gases are used which have appreciable substitution of carbon for oxygen, but are otherwise tissue equivalent.^(5, 6)

The cross-section data used in this computation have been taken from the following sources:

Hydrogen	Isotropic, elastic cross sections, Reference 7.
Carbon	0-5 MeV, Reference 2. Total cross sections, no nonelastic contribution. 5-10 MeV, Reference 2. Total cross sections minus nonelastic contribution.
Nitrogen	0-0.88 MeV, Reference 2. Total cross sections, no nonelastic contribution; 0.88-10.0 MeV, Reference 4. Elastic cross-section values from graph.
Oxygen	0-1.7 MeV, Reference 2. Total cross section, no nonelastic contribution; 1.7-3.2 MeV, Reference 4. Elastic cross-section values from graph. 3.2-10.0 MeV, Reference 2. Total cross sections minus nonelastic terms as follows: n, α cross sections 3.6-5.2 MeV, Reference 8; 5.0-8.5 MeV, Reference 2; n, γ cross sections 6.5-10.0 MeV, Reference 7.

The coefficient of anisotropy, $K_i(E)$ was obtained

FIGURE 7

FIGURE 8

FIGURE 9

FIGS. 7-9.—Absorbed dose in pure carbon, nitrogen and oxygen from a fluence of 10^9 neutrons per cm^2 , as a function of neutron energy.

by numerical integration of the angular distribution curves in Reference 3. At energy values not covered in this publication values of $K_i(E)$ were obtained by interpolation. Total cross-section data were taken at 0.1-MeV energy intervals with additional points in

regions where the cross section was changing rapidly with energy.

The data presented in Figures 7-10 for the pure elements H, C, N, and O has been transferred to punched cards.

FIG. 10.—Absorbed dose in tissue (ICRP Standard Man), and pure hydrogen (dose scale $\times 10^{-1}$) from a fluence of 10^9 neutrons per cm^2 , as a function of neutron energy.

CONCLUSION

It is now possible, by the use of simple computer programming, to determine the dose for any tissue composition and any neutron spectrum with minimum effort. It is planned to extend the scope of the data to include the elements calcium, phosphorus, and argon so that various bone compositions can be accommodated, in addition to the characteristics of argon-filled ionization chambers⁽⁵⁾ such as are used for gamma dosimetry in the presence of neutrons.

Early publication of these data, with composition-correction factors for tissue-equivalent materials in common use, is expected.^(6, 9)

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ABSOLUTE DOSIMETRY BY CALORIMETRIC MEASUREMENTS

Paul Mitacek, Jr.

PURPOSE AND METHODS

The purpose of this project is to provide a method of measuring absorbed dose absolutely for both γ -rays and neutrons. At present calorimetry provides the only method of *directly* measuring neutron absorbed doses. Calorimetry will be used to make neutron absorbed-

dose measurements of radiation fields from the Janus reactor.

PROGRESS REPORT

Measurements of absorbed dose in a carbon calorimeter were made with the low-temperature calorimeter

system Mark III,⁽¹⁾ using gamma rays from the Co⁶⁰ high-level radiation room. A carbon calorimeter was used in order to eliminate an endothermic chemical bond-breaking effect⁽²⁾ which occurs in many materials when exposed to ionizing radiation. The endothermic chemical bond-breaking effect, if present, would produce an error in the absorbed-dose measurement which may be difficult to assess.

The calorimeter was not completely adiabatic during the irradiations because of the inability of the vacuum system to maintain a vacuum of 10^{-7} mm of Hg vapor pressure or lower. This resulted in a thermal cold drift in the calorimeter for which a correction had to be made. This correction amounted to 3% of the total heat rise in the calorimeter for each exposure.

The absorbed-dose measurements were made at a distance of 1.00 meter. The dose rate inside the cryostat at that distance was 1.998 rads per second. The standard deviation of the individual measurements was 0.6% of the arithmetic mean. The standard deviation of the mean was 0.2% of the arithmetic mean.

A parallel-plate, carbon-free-air ion chamber was constructed with an electrical guard ring around the collecting electrode. The ion chamber replaced the calorimeter inside the cryostat for the ion-chamber measurements. Current readings from the ion chamber were made under the same radiation conditions and at the same distance from the Co⁶⁰ source as was the calorimeter during the calorimetric measurements. The current from the ion chamber was measured with a Cary 31 vibrating reed electrometer to an accuracy of better than 1%.

The ion chamber provides a means of indirectly comparing the calorimetric measurements with similar calorimetric measurements of other investigators. The Bragg-Gray relation⁽³⁾ is

$$D = W \cdot J \cdot s,$$

where D is the energy absorbed per gram of carbon, J is the number of ion pairs formed per gram of air in the ion chamber, s is the mass stopping ratio of carbon to air, and W is the energy required to form each ion pair in air. The energy absorbed per gram of carbon is obtained from the calorimetric measurements. The number of ion pairs formed, J , is calculated from the ion chamber measurements. The value of s has been calculated and published.⁽⁴⁾ A value for W for air was thus calculated and found to be 33.91 eV. The weighted mean value for W in air of a number of independent investigators is 33.73 ± 0.15 eV.⁽⁴⁾ My value agreed

with this average to within the limits of error. Plans have been made to make a direct calorimetric comparison with Dr. John S. Laughlin and his group at the Sloan-Kettering Institute for Cancer Research. The

dynamic liquid helium probe cryostat measurements will be compared with the carbon calorimeter measurements of the Sloan-Kettering group.⁽²⁾

In preparing for the neutron absorbed dose measurements on the Janus reactor, it became evident that additional measurements and subsidiary studies, such as measuring chemical bond-breaking effects in tissue-equivalent plastics,⁽¹⁾ would be necessary. The low-temperature calorimeter system Mark III is adequate for measurements at Janus. However, because of its size and because of geometrical considerations, it could not be used for measurements at the Van de Graaff accelerators in Buildings 203 and 208 and the cyclotron in Building 211. An improved and more flexible cryostat system adaptable to measurements at the various radiation facilities was designed and constructed.

Figure 11 is a labeled cut-away drawing of the dynamic liquid-helium probe cryostat assembly and calorimeter. The major improvements of this cryostat over the previous model are as follows:

- 1) The calorimeter can be placed within an inch of an external radiation source. This proximity to an external source is necessary in order to have measurable dose rates from the monoenergetic neutron beams produced by the $\text{Li}^7(p,n)\text{Be}^7$ reaction on the targets of the various Van de Graaff accelerators.⁽⁵⁾

- 2) The calorimeter holder is fixed in machined grooves so that the calorimeter can be positioned with an accuracy of 0.01 of an inch to an external point. This accuracy in positioning is necessary for measurements close to the radiation source.

- 3) The cryostat is constructed to operate in a horizontal plane. This allows it to be used as a probe unit and be placed in radiation beam ports such as that at the cyclotron.

- 4) By proper design of the vacuum pumpout system improved vacuum and greater pumping speed was obtained. A 2-inch oil diffusion pump was placed on the cryostat for greater pumping efficiency. The increased isolation of the calorimeter allows longer exposure times so that lower dose rates can be measured without any decrease in precision.

Operation of the dynamic liquid helium probe cryostat differs from the operation of the low-temperature calorimeter system Mark III.⁽¹⁾ Liquid helium and liquid nitrogen are forced, by gas pressure in the storage dewars, through the helium and nitrogen input tubes, respectively. The rate of flow of the liquids, and thus the temperatures inside the cryostat, is controlled by the flow of gas out of the gas-throttling valves. The calorimeter is cooled to the temperature of the liquid-He shield by the heat-transfer medium of the helium gas. The calorimeter is isolated after being cooled by pumping out the helium transfer gas to a

DYNAMIC PROBE CRYOSTAT

FIG. 11.—Dynamic liquid-helium probe cryostat assembly and calorimeter

vacuum of 10^{-7} mm of Hg vapor pressure or better. The measurement of absorbed doses with the calorimeter is thereafter identical with the procedures in the low-temperature calorimeter system Mark III.⁽¹⁾

The dynamic liquid-helium cryostat has been operated at liquid-helium temperatures and some preliminary tests performed using a carbon calorimeter. Later, a tissue-equivalent calorimeter will replace carbon for γ -ray and neutron measurements. The temperature-measuring circuit and electrical-heating circuit⁽¹⁾ have been improved with continuous-recording electronic instrumentation. The accuracy of the temperature measurements will not be the limiting factor in the absorbed dose measurements.

CONCLUSIONS

The sensitivity and accuracy of the low-temperature calorimetric absorbed-dose measurements have been

demonstrated. Neutron absorbed dose measurements in tissue can be made with the same sensitivity; their accuracy will depend upon the accuracy of the correction term for the endothermic bond-breaking effect.

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THE CALIBRATION AND USE OF NaI(Tl) SYSTEMS

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PURPOSE AND METHODS

Although the common NaI(Tl) gamma scintillation system is known to be nonlinear in its response to photon energy,⁽¹⁻⁶⁾ such linearity would be very desirable, especially in biological work. In such work, too, distributed sources are very common. But our experience (*vide infra*) showed that calibration depends on both the geometry and composition of such sources in a manner not appreciated in studies reported previously.

Accordingly, we have attempted during the past year to develop linear NaI (Tl) systems and convenient calibration sources and methods applicable to distributed systems. Our method was simply the careful cross calibration of a large number of known sources of varied energy, composition, and geometry with one another and with various crystals and analyzers.

PROGRESS REPORT

Although a large number of systems was tested, results were so similar that a few examples will illustrate our findings. The data presented were obtained with a 4" x 4" NaI(Tl) crystal in a low-background shield using a 400-channel analyzer* and calibrated signal generator.†

* Allen B. Dumont Co. Type 6292; Radiation Instrument Development Laboratories Model 31-15; Radiation Instrument

Although simple linearity cannot be expected from such a system, we found that the *deviation* is itself linear above 250 keV. Thus by adjusting system constants to

$$Y = 0.105 m - 3.3$$

we were able to obtain a response

$$Y = 0.100 m$$

for energies above 250 keV. Here, Y is the photopeak channel determined by the method of Onno⁽⁷⁾ and m the photopeak energy.

Results are shown in Table 4, and linearity can be seen to be applicable within ± 4 keV above $m = 250$ keV for single photopeaks. For some photopeaks, i.e., those which appear because of the coincidence summation of two single lines, discrepancies are much larger. This is to be expected from the nature of the crystal response⁽¹⁻⁶⁾ and points up the danger of using sum peaks for calibration, as is often done.⁽¹⁾

Because no radionuclide gamma sources of sufficiently high energies and convenient half-life are commonly available, we developed an equivalent system. A solution 1 M in $\text{Th}(\text{NO}_3)_4$, 0.1 M in $\text{Pb}(\text{NO}_3)_2$ and 0.1 M in $\text{Bi}(\text{NO}_3)_3$ subjected to rapid chloride precipi-

Development Laboratories Model 34-12B; Hewlett-Packard Co. Model 212-A.

† Hewlett-Packard Co. Model 212-A.

TABLE 4. PULSE HEIGHT-ENERGY RESPONSE FOR STANDARD GAMMA ENERGIES

Nuclide	Gamma energy, keV	Peak type	Observed photopeak, equivalent keV	Error, equivalent keV
Pb ²¹² *	77	Single	57	-20
Cd ¹⁰⁹ Ag ^{109m}	88	"	70	-18
Fe ⁵⁹	145	"	119	-26
Fe ⁵⁹	191	"	174	-17
Pb ²¹² *	238	"	232	-6
Fe ⁵⁹	336	Sum	320	-16
Be ⁷	478	Single	480	+2
Na ²²	511	"	513	+2
Zn ⁶⁵	511	"	512	+1
Tl ²⁰⁹ *	584	"	585	+1
Cs ¹³⁴	605	"	602	-3
Cs ¹³⁷	662	"	660	-2
Cs ¹³⁴	797	"	799	+2
Mn ⁵⁴	842	"	841	-1
Mn ⁵⁶	845	"	844	-1
Sc ⁴⁶	887	"	886	-1
Fe ⁵⁹	1097	"	1094	-3
Zn ⁶⁵	1119	"	1122	+3
Sc ⁴⁶	1119	"	1120	+1
Co ⁶⁰	1173	"	1172	-1
Na ²²	1277	Single	1276	-1
Fe ⁵⁹	1289	Single	1286	-3
Co ⁶⁰	1333	"	1332	-1
Na ²⁴	1368	Single	1367	-1
Cs ¹³⁴	1402	Sum	1423	+21
K ⁴⁰	1460	Single	1460	0
Al ²⁸	1780	"	1776	-4
Na ²²	1785	β^+ Sum	1821	+36
Cs ¹³⁴	1970	Double sum	2115	+45
Sc ⁴⁶	2006	Sum	2030	+24
Co ⁶⁰	2506	"	2536	+30
Tl ²⁰⁸ *	2615	Single	2616	+1
Na ²⁴	2754	"	2751	-3
Tl ²⁰⁸ *	3198	Sum	3222	+24
Na ²⁴	4212	"	4262	+50

* As seen in the spectrum of natural Th.

tation of Pb²¹² followed by oxychloride precipitation of Bi²¹² provided a very convenient gamma-calibration source. The final product contained Bi²¹² and Tl²⁰⁸ with just enough Pb²¹² to provide convenient lines at 77, 238, 584 and 2615 keV and an effective half-life of about 6 hours. Samples were prepared in a few minutes from stock solution at very low cost and with a high degree of reproducibility, not only as to gamma energy but as to activity as well. With this source, it was relatively simple to go from stock solution to complete calibration of the system in about 30 minutes.

Geometric effects on apparent gamma energy were found which depended on the position or distribution of the source. From the representative data given in Table 5, one can see that these effects could easily

amount to ± 30 keV for the common biological situation of counting animals or samples poured into re-entrant counting cups. This effect does not seem to be explicable in terms of present theory and is under study. Operationally, however, we have been able to bypass it by distributing standards in suitable phantoms for calibration. In this connection, we have found that ordinary reagent grade KF, dried at 110° for an hour or so, makes a much more useful K⁴⁰ standard, because of its higher K content and analytical purity, than the K salts now commonly used at various other laboratories.

We have also found that total efficiency and, to a smaller degree, photopeak efficiency is dependent on matrix composition. Thus, aqueous solutions of KF, KCL, KBr and KI of the same molar concentration

TABLE 5. APPARENT PHOTOPEAK ENERGY AS A FUNCTION OF SOURCE GEOMETRY

Nuclide	Source		Gamma energy, keV	Observed photopeak, keV	Error, keV
	Type*	Position†			
Cs ¹³⁷	a	top	662	660	-2
	a	side	662	650	-12
Co ⁶⁰	a	top	1173	1172	-1
	a	side	1173	1158	-14
	a	top	1133	1332	-1
	a	side	1133	1317	-15
	a	top	2506	2536	+30
	a	side	2506	2486	-20
Fe ⁵⁹	a	top	145	119	-26
	a	side	145	114	-31
	a	top	191	174	-17
	a	side	191	168	-23
	a	top	336	320	-16
	a	side	336	290	-46
K ⁴⁰	a	top	1097	1094	-3
	a	side	1097	1083	-14
	a	top	1289	1286	-3
	a	side	1289	1274	-15
	b	top	1460	1460	0
	b	side	1460	1445	-15
Na ²²	c	—	1460	1451	-9
	a	top	511	513	+2
	a	side	511	497	-14
	a	top	1277	1276	-1
	a	side	1277	1265	-12
	a	top	1785	1821	+36
Na ²⁴	a	side	1785	1786	+1
	a	top	1368	1367	-1
	a	side	1368	1348	-20
	a	top	2754	2751	-3
	a	side	2754	2725	-29

* a = disc source, 1 cm diameter by 50 μ thick; b = cylindrical source, 454 g KF, 75 cm diameter by 75 cm high; c = inverted reentrant cup, 2235 g KF, 2.5 cm thick.

† top = top dead center; side = at crystal surface, centered on midplane.

gave different total and photopeak efficiencies, as did solutions of the same salt at different concentrations. As might be expected from theory, the ratio of photopeak to total efficiency decreased with increasing Z of the matrix.

This effect could be mitigated, as above, by the use of gel phantoms⁽⁸⁾ but not completely eliminated. Such elimination will probably require incorporation of synthetic bones, air spaces, etc.

In an attempt to find alternatives to the expensive low-background shields currently used in biological work, we surveyed a number of possible types of "junk-pile" equipment. All-steel safes built prior to 1945 were found to make very useful low-background shields and were available at very low cost from second-hand safe dealers.

CONCLUSIONS

1. Although the response of NaI(Tl) gamma scintillation crystals is inherently nonlinear an approximation good to $\pm 0.1\%$ for the linear equation

$$Y = mx + 0$$

can be obtained for energies above 250 KeV by adjusting the system to

$$Y = m'x + b$$

where m' is $> m$ and b is negative.

2. A convenient calibration source, derived from natural Th, is described which avoids the problems inherent in the use of sum peaks.

3. Two geometric problems are described, the dependence of efficiency on matrix composition and the shifts of photopeak apparent energy and resolution by distributed sources. Methods of dealing with them are described.

4. All-steel safes constructed prior to 1945 have been found to provide useful low-background shields.

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FERROUS AND CERIC DOSIMETERS FOR 50-1500 RADS

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PURPOSE AND METHODS

Partly because of their nature and partly because of the extensive data available on their characteristics, the classical ferrous and ceric sulfate systems would provide nearly ideal dosimeters for mixed-field problems such as those attending the Janus program.⁽¹⁾ Unfortunately, they are useful only above 2000 rads, a range of relatively little biological interest. We thus began attempts to increase their sensitivity by at least an order of magnitude without changing the irradiated systems themselves so as to maintain reference to the large body of data available for the classical systems.

We had previously observed that use of very high concentrations of SCN^- would increase the sensitivity of the ferrous sulfate markedly over the best previously obtained.⁽²⁻⁴⁾ We had also observed that the quantitative oxidation of fluorescein by ceric ion could be made the basis of a very sensitive fluorometric ceric

dosimeter.⁽⁴⁾ We, therefore, attempted, this year, to reduce these dosimetric systems to practice.

PROGRESS REPORT

Ferrous Sulfate

By addition of a saturated aqueous solution of NH_4SCN to an aliquot of the usual ferrous dosimeter ($10^{-3} M$ in $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$, $10^{-3} M$ in NaCl , $0.4 M$ in H_2SO_4), we were able to promote the production of a deep red complex of high extinction coefficient ($\epsilon_{475} = 17,600 \text{ liter mole}^{-1} \text{ cm}^{-1}$). Double glass-distilled water proved an adequate solvent, and irradiations were performed in 30-ml polyethylene bottles prerinsed with dosimeter. We have not been able to observe a change of ϵ_{475} with temperature over the range of $20-25^\circ\text{C}$, nor with time from 5 to 25 minutes.

In practice, 1.0 ml of saturated aqueous NH_4SCN was added to 3.0 ml of irradiated dosimeter solution

FIG. 12.—Ferrous sulfate-thiocyanate system, 1 cm. Dosimeter solution $10^{-3} M$ in $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$, $10^{-3} M$ in NaCl and $0.4 M$ in H_2SO_4 , irradiated in 30-ml polyethylene bottles at a midline, air dose rate of 102.5 R min^{-1} , 250 KVP x-rays, 0.5 mm Cu plus 1.0 mm Al. 3.0 ml-aliquots of irradiated solution were removed in duplicate, treated with 1.0 ml saturated aqueous NH_4SCN and read at $475 \text{ m}\mu$ in 1.0-cm cuvettes 5 to 20 minutes after addition, 23.5°C , against unirradiated solution.

FIG. 13.—Ferrous sulfate-thiocyanate system, 5 cm. Dosimeter solution $10^{-3} M$ in $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$, $10^{-3} M$ in NaCl , $0.4 M$ in H_2SO_4 , irradiated in 30-ml polyethylene bottles at a midline, air dose rate of 80.4 R min^{-1} , 250 KVP x-rays, 0.5 mm Cu plus 1.0 mm Al. 15.0-ml aliquots removed, treated with 5.0 ml saturated aqueous NH_4SCN and read in 5.0-cm cuvettes at $475 \text{ m}\mu$ 5 to 20 minutes after addition, 23.5°C , against unirradiated solution.

and the resultant mixture read at $475 \text{ m}\mu$ at $20\text{--}25^\circ\text{C}$, 5 to 25 minutes after addition, against a blank containing unirradiated dosimeter solution. As a routine precaution, at least two aliquots were taken and the results averaged. A typical run is shown in Figure 12.

Runs were usually read in a 3-ml, 1.0-cm cuvette on

a Beckman Model DU spectrophotometer. If the volume of dosimeter was limited, the use of 0.75 ml dosimeter plus 0.25 ml NH_4SCN in a 1-ml cuvette gave identical results, although more care was needed in handling the smaller volumes.

For greater sensitivity, 15 ml of dosimeter plus 5 ml of NH_4SCN was used in a 15-ml, 5.0-cm cuvette. The results of such a run are shown in Figure 13.

A series of runs made with known amounts of Fe^{3+} added to unirradiated dosimeter showed the inherent precision to be about $\pm 0.2\%$. The precision reflected in Figures 12 and 13 is not quite so high. However, comparative dosimetry was done with calibrated Victoreen chambers whose own precision was only of the order of $\pm 1\%$ so that the discrepancy observed was probably due to inadequate dose control.

As is evident from the figures, Beer's Law is followed closely so that one can obtain the Fe^{3+} concentration directly using $\epsilon_{475} = 17,600 \text{ liter mole}^{-1} \text{ cm}^{-1}$. This, adjusted for the G value appropriate to the radiation employed, then gives dose directly.

Ceric Sulfate

Ferrous ion is thermodynamically unstable with respect to oxidation by air, and a slow autoxidation is always noted with ferrous sulfate systems even in the absence of radiation. Ceric ion is stable in this respect but unstable with respect to reduction by water. This reduction proceeds appreciably only in the presence of radiation or strong light so that ceric solutions should

be handled in red or opaque glass containers. Ordinary room light may be tolerated for periods of a few hours without observable effect so that light does not present a problem in ordinary handling.

The chief source of instability with ceric solutions is the light-catalyzed oxidation of trace metals or organic materials present in all but the very cleanest of surfaces.⁽⁵⁾ While this is a nuisance in dosimetry, the effect itself can be used to eliminate its own consequences. Thus, we clean our glassware by filling it with ceric solution and either warming it or exposing it to strong light (e.g., a Sylvania Corp. Sun Gun) for an hour or so as convenience dictates. The solution is then drained out and fresh dosimeter solution added. The small amount of partly reduced solution remaining on the walls proved to have an insignificant effect (i.e., <0.01%) on the over-all measurements.

Dosimeter solution itself is made up a few per cent more concentrated than needed and illuminated for a few moments within its storage flask (glass stoppered) before wrapping the flask in a foil for dark storage. Determinations of ceric concentration showed no (i.e., <0.1%) change over a period of several months when stored in this way.

At first a $10^{-5} M$ solution of Ce^{4+} in $0.4 M H_2SO_4$ was irradiated and then duplicate 12.5-ml aliquots removed. Each aliquot was added to 1.0 ml of $5 \times 10^{-5} M$ aqueous sodium fluorescein, 5 minutes allowed for oxidation, then 1.5 ml of $19 M NaOH$ added to bring the unoxidized fluorescein into anionic form. Readings were taken in a simple Fisher Nephfluorometer at an

FIG. 14.—Ceric dosimetry, Fisher Nephfluorometer. Dosimeter solution $10^{-5} M Ce^{4+}$ in $0.4 M H_2SO_4$ irradiated in covered, glass vials at a midline, air dose rate of $123.2 R min^{-1}$, 250 KVP x-rays, 0.5 mm Cu plus 1.0 mm Al. 12.5-ml aliquots removed and determined as per text.

FIG. 15.—Ceric dosimetry, Turner Fluorometer. Dosimeter solution $10^{-5} M Ce^{4+}$ in $0.4 M H_2SO_4$ added in known proportion to $0.4 M H_2SO_4$ and 12.5-ml aliquots removed for analysis as per text, where $X = (KN_f - N_c)$.

excitation wavelength of $365 m\mu$ and an emission wavelength of $430 m\mu$. Results of a typical run are presented in Figure 14.

It was evident that this method, while sensitive and precise enough ($\pm 5\%$) for many uses, was not sufficiently precise for our purposes. At least part of the difficulty lay in the fact that the instrument had a nonlinear scale whose readability was limited to $\pm 2\%$.

Accordingly, we obtained a more sensitive and sophisticated instrument, the Turner Model 101 Fluorometer and equipped it with a Vernier scale, permitting readings to $\pm 0.1\%$. At the same time, it became evident that residual fluorescence was probably not a simple function of ceric concentration so that a more sophisticated equation was also needed for investigations of precision, if not for dosimetry. This yielded a theoretical equation which, for constant final solution mass, may be written

$$Y = f(X) + b = f(KN_f - N_c) + b$$

where Y is the instrument reading in fluorescence dial units, N_f and N_c the moles of fluorescein and Ce^{4+} , respectively, K the number of moles of ceric ion required to oxidize one mole fluorescein, and b a system constant.

With the Turner instrument at excitation = $405 m\mu$, emission = $510 m\mu$, sensitivity was markedly better than that previously obtained with the Fisher. By titration, K was found to be 2.26. The results of a typical run using $K = 2.26$ are shown in Figure 15. This run was done with known, added quantities of Ce^{4+} rather than with radiation, as a check on the in-

herent precision of the method. While adequate, it still does not equal the levels thought feasible (i.e., $\pm 0.1\%$) so that further work in this direction will be done before returning to irradiation tests.

Since fluorescein itself has a quantum fluorescent efficiency of the order of 70%,⁽⁶⁾ no substance of very much higher effective sensitivity is possible. Under present conditions, we are utilizing only about 1% of the sensitivity of which the fluorescein and machine are capable. Thus, the present maximum sensitivity of about 50 rads could easily be extended to 1 rad or less were the precision adequate.

CONCLUSIONS

1. A sensitive, convenient, and rapid system for ferrous-sulfate dosimetry is described utilizing $\text{NH}_4\cdot\text{SCN}$ which is usable in the range 50–1500 R with precisions of the order of $\pm 0.5\%$.

2. A fluorometric system of ceric sulfate dosimetry is described which is usable in the range 50–1500 rads and, potentially, in the range 1–1500 rads. However,

problems of precision for this method are still to be solved.

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Somatic Effects

THE GENETIC FACTOR IN THE RELATION OF RADIATION RESISTANCE AND LONGEVITY TO BRAIN STRUCTURE AND BEHAVIOR IN LABORATORY MICE

George A. Sacher, Dale D. Morris, and Frank Verley

PURPOSE AND METHODS

This experiment was designed to estimate the genetic correlations of longevity and radiation resistance with certain behavioral traits and with brain structure, using five inbred strains of mice and their hybrids.

The genetic design is basically a diallel table with

TABLE 6. MATING PLAN FOR DIALLEL ANALYSIS OF THE RELATION OF LONGEVITY TO BRAIN STRUCTURE AND BEHAVIOR

The Cell Entries are the Short Codes for the Progeny of the Various Matings

Sires	Dams				
	A/Jax	Balb/c	C57BL/6	C3H/f	DBA/1J
A/Jax	A	CA	BA	HA	DA
Balb/c	AC	C	BC	HC	DC
C57BL/6	AB	CB	B	HB	DB
C3H/f	AH	CH	BH	H	DH
DBA/1J	AD	CD	BD	HD	D

5^2 possible crosses or subclasses. An analysis of variance for the diallel table and a description of the genetical situation associated with diallel tables are given by Hayman.⁽¹⁾ The analysis of variance essentially tests additive and dominance effects obtained from the progeny of the diallel cross. The strains used and the mating plan for this experiment are given in Table 6. In addition to the genotypes of Table 6, an equal number of C57BL/10 mice will be studied to determine whether the establishment of independent sublines leads to an appreciable amount of genetic drift in the anatomical and physiological characters of interest to us.

All young are weaned at 30 days of age, and assignments to experimental groups are made at weaning. Longevity will be estimated using 24 animals of each sex and genotype. Since mice must be housed 10 to 12 per cage for economic reasons, the usual practice of caging different genotypes separately would almost certainly yield worthless results. We have, therefore, adopted a design in which each cage of 10 mice is set

up at weaning with 2 or 3 mice of each of 4 or 5 different genotypes represented. Since there are 15 different genotypes (ignoring reciprocal crosses) and 72 cages, an incomplete balanced design can be set up. Thus far, the mixing of genotypes has created no problems of disease or fighting, but the performance of these cages continues to be watched carefully. If this design proves to be successful, it will be made a basic feature of the studies of acute and long-term radiation effects when these phases of the program are begun.

Brain weight will be the first neuroanatomical parameter to be examined. Brain weight, body weight, body, tail and hind foot lengths, and liver, spleen, kidney, adrenal, heart and testis weights will be taken. Sample size will be 24 of each genotype-sex subclass. Brains are fixed for ultimate sectioning and determination of sizes of major brain structures. The anatomical data will be subjected to variance-covariance analysis as a preliminary to comparisons with the physiological and psychological traits to be measured. A subgroup of mice will be put through behavioral tests and then sacrificed, so that the correlation of brain weight with performance can be estimated. This, however, will be done on a segregating F_2 or subsequent generation.

A battery of behavior tests will be developed and administered to each subclass of the diallel design in an attempt to arrive at an over-all behavioral score which can then be correlated with the genotype brain and organ weights.

Now under way is a behavior study employing a maze described by Maier and Schneirla.⁽²⁾ This device allows scores for original learning and for relearning to be determined by using different starting positions in the same maze. Ninety-day-old animals are maintained at 80% *ad lib.* body weight and run with food pellets as reinforcement. Animals are given three trials

TABLE 7. MEAN BRAIN WEIGHT* IN GRAMS

	C	H	CH	D	A	B
Male	0.487	0.491	0.496	0.407	0.425	0.455
Female	0.500	0.487	0.516	0.407	0.427	0.449

* N = 20.

per day. The criterion used to demonstrate that learning has occurred is three successive errorless trials. Data are being recorded as number of errors per trial and time duration of each trial. Data will be analyzed in terms of total errors, total trials, and mean time required to reach criterion.

Studies will also be carried out which utilize instrumental conditioning techniques. Animals are maintained at 80% *ad lib.* body weight and trained to press a lever in the conditioning box for 16% sucrose solution as reinforcement. This technique yields a very stable baseline in terms of lever presses which can be used to assess the effects of other variables introduced into the situation. Data can be analyzed in terms of a signal-to-noise ratio.

Currently being initiated is a study of the radiation sensitivity of the various subclasses of mice utilizing a technique developed by Morris.⁽³⁾ Superimposed upon the baseline is a 15-second burst of x-irradiation at 0.5 R/sec followed by a brief electric shock intense enough to be noxious to the animal. The noxious quality of the shock is associated with the x-irradiation exposure and bar pressing ceases during irradiation after several pairings of x-ray and shock. Dose-rate can then be systematically decreased and a threshold value determined. This technique has been used to demonstrate that Sprague-Dawley rats can discriminate the presence of x-rays at a dose rate as low as 4 mR/sec. Animals blinded by bilateral ophthalmectomy can make this discrimination as rapidly as normal animals.

Instrumental conditioning techniques will also be used to measure escape-avoidance behavior and visual and auditory discriminations.

PROGRESS REPORT

The results to date are presented in Figure 16 and Table 7. Figure 16 shows the trials to criterion for learning the initial maze pattern and for relearning two altered patterns. The results indicate that there are two or more distinct parameters for learning and relearning, and that the genetic differences in these parameters may be appreciable. The results are too preliminary to be interpreted in detail at this time.

FIG. 16.—Mean trials to criterion for the initial Maier and Schneirla maze pattern (1 on abscissa) and for two altered patterns (2 and 3 on abscissa).

Table 7 gives a sample of the early and incomplete brain-weight study. It can be seen that strain differences are large compared to sex differences within genotype. The variance between individuals within genotype is small enough so that sex and genotype differences of the magnitude seen here are highly significant.

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SURVIVAL OF MICE UNDER DAILY GAMMA IRRADIATION: PROGRESS REPORT

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

PURPOSE AND METHODS

The initial studies on chronic radiation lethality under duration-of-life daily gamma irradiation were limited in their ability to obtain adequate data at doses below 12 R/day.⁽¹⁾ A second series of exposures was, therefore, begun in 1958 in an effort to improve our

knowledge of low-dose responses. This series reached final dying out within the last year. The study was briefly described in an earlier progress report.⁽²⁾ It has employed comparatively large numbers of mice of both sexes of inbred strains A/Jax, BALB/c and C57BL/6 and the hybrid BCF₁ (C57BL × BALB/c). Proportionality of sample sizes was maintained for dose levels of 0, 0.3, 1.3, 2.6, and 6 R/day. At dose levels of 12, 24, 32, 43, and 56 R/day, equal samples were employed for all strain and sexes within each dose group. The exact plan was given in the prior report,⁽²⁾ and the numbers actually achieved after removal of animals for causes not ascribable to the experiment (i.e., accidental death, missing animals, etc.) are given in Table 8. No serious imbalances occurred as a result of this intercurrent loss, and it is, therefore, possible to achieve one of the original goals—a proportionately weighted combination of all strains, within and across sex and within dose levels, so as to attain statistics on groups of 500 and more animals. This report presents the first summary of the completed lethality data. Pathological studies are not yet complete.

TABLE 8. SAMPLE SIZES USED TO DERIVE MAS VALUES

Strain	Sex	Daily exposure dose, R/day									
		0	0.3	1.3	2.6	6	12	24	32	43	56
A/Jax	♂	82	60	82	47	48	46	46	23	22	24
	♀	83	54	83	48	48	47	48	24	24	24
BALB/c	♂	77	57	82	47	46	46	45	24	23	23
	♀	84	59	84	47	47	46	48	23	24	24
C57BL/6	♂	82	—	83	48	47	46	48	24	22	24
	♀	82	—	83	46	47	45	48	23	23	22
BCF ₁	♂	165	120	164	96	96	48	48	24	24	23
	♀	166	116	167	96	95	47	48	24	24	24

TABLE 9. 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
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
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 ± 1
BCF ₁	♂	710 ± 15	762 ± 18	689 ± 12	698 ± 17	599 ± 12	467 ± 17	301 ± 12	207 ± 8	166 ± 5	99 ± 5
	♀	796 ± 14	815 ± 14	722 ± 9	657 ± 15	580 ± 11	448 ± 12	282 ± 12	212 ± 11	160 ± 5	92 ± 3

PROGRESS REPORT

The mean after-survival (MAS) values are given in Table 9 by sex, strain, and dose groups with the appropriate standard errors of the mean. The MAS values are all based on a starting age of 100 days. Figure 17 plots the log MAS values for combined sexes against arithmetic daily dose, a relationship that we previously observed to be an essentially linear one and that was also observed to be nearly invariant among strains and hybrids.^(1, 3) This linearity is again seen here, and in several strains, the fit is almost perfect over a broad daily-dose range. The regression coefficients have not yet been precisely derived, but first approximations indicate the slopes vary from -0.037 to -0.043 R/day. These values are slightly higher than previous figures of -0.033 for the LAF_1 ⁽¹⁾ and -0.038 for the average of six different strains and hybrids,⁽³⁾ including the four of the present study. The estimates were not from concurrent experiments and probably reflect progressive changes in animal husbandry techniques over the decade 1954-1964. MAS values at the higher dose levels are very similar to those seen in the previous study,⁽⁴⁾ but definite im-

FIG. 17.—Log mean after-survival versus daily-dose relationship for combined sexes of the indicated strains. MAS values measured from starting age of 100 days.

FIG. 18.—Age-specific mortality rate curves for females from the combined data of strains A/Jax, BALB/c, C57BL/6 and the BCF_1 hybrid.

provement in survival is noted at the low dose levels. The obvious effect is to steepen the slopes.

The data at 0.3 R/day have not been included, since these mice were caged differently and a careful comparative analysis of the mortality distributions for these animals along with those at 0, 1.3, and 2.6 R/day is required. The flattening of the MAS vs. daily dose relation for the A mice at doses below 6-12 R/day also requires thorough pathological and statistical evaluation. The effect may be attributable to the high spontaneous incidence of a chronic degenerative disease of the parenchymatous organs.

The age-specific mortality rates for doses up to 24 R/day are given in Figures 18 and 19. The sexes are presented separately, but the strains are combined within dose levels. The females show a nearly ideal array of mortality curves; there is a progressive increase in slope with increase in daily dose and thus a progressive divergence of the irradiated population from the controls with increase in age and accumulated injury.

The males (Figure 19) do not show the same ideal picture. At the three lowest doses, there is little clear separation from the controls for the first 400-500 days,

FIG. 19.—Age-specific mortality rate curves for males from the combined data of strains A/Jax, BALB/c, C57BL/6 and the BCF₁ hybrid.

but a dose-dependent divergence does occur late in life. The MAS values in Table 9 also reveal that most of the male populations do not demonstrate a significant life shortening below 6 R/day. Only one instance

of over-survival occurred (BCF₁ at 0.3 R/day), which suggests the persistence of a fairly stable environment and low intercurrent infectious disease rate during the course of the study, since over-survival has generally been associated with suboptimal experimental conditions.

The control mortality rate curves show one feature that is somewhat atypical. Beyond 600 to 700 days, there is a definite drop in the rate of the exponential increase in mortality that is to some degree paralleled by the 1.3- and 2.6-R/day groups. Although this drop in death rate is sometimes seen in human and animal populations at advanced ages, its presence here may be at least partly due to the combining of data across strains rather than an innate quality of the data.

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THE COMPARATIVE LETHAL EFFECTS OF PRENATAL AND POSTNATAL DAILY IRRADIATION

Douglas Grahn and Katherine F. Hamilton

PURPOSE AND METHODS

Although a number of studies have detailed the detrimental effects of prenatal irradiation on the developing embryo and fetus, little effort has been made to establish the level of chronic radiation injury induced. The positive evidence presented by Stewart *et al.*⁽¹⁾ and MacMahon⁽²⁾ on the induction of malignant tumors in children by a single pelvimetric x-ray examination during late gestation has emphasized the need for data, since these human studies suggest the fetus to be particularly sensitive to somatic injury.

Our own effort is designed to examine the effects on

mice of low daily doses of irradiation delivered during gestation in comparison with exposures limited to several postnatal periods. Low daily exposures are employed to avoid the induction of gross anomalies. The plan of the experiment is given in Table 10. The experiment uses both sexes of the C57BL/6 mouse, chosen for its sensitivity to the induction of leukemia. A parent female is used only once to avoid any complicating mutational effects.

The groups I, II, and III are expected to determine the specific effects of prenatal irradiation only compared to postnatal only and the whole lifetime. Group

IV is our standard age group for chronic toxicity studies and will permit cross reference to the extensive experience at doses ranging from 0.3 to 56 R/day (see previous article). Three replications have been set up with equal numbers of breeding females per group. The first two provided inadequate sample sizes owing to fertility problems even in the absence of radiation. All progeny are first parity mice in those replications. The third replication used only proven dams and sires, thus producing second parity animals for the experiment. No additional replications are planned until the data warrant increases in sampling or, if necessary, inclusion of either higher or lower dose levels. Sample sizes, at weaning, for the five groups are:

Group:	2.6 R	6 R
I	98	70
II	82	56
III	82	99
IV	110	110
V	—	99

The inequalities reflect radiation effects on both the number born and the survival to weaning.

PROGRESS REPORT

No significant survival statistics are yet available regarding long-term injury, but the reproductive performance data do reveal that the dose levels are probably near optimal regarding the avoidance of severe prenatal and early postnatal losses.

The litter sizes at birth are:

No prenatal irradiation (III, IV, V):

6.32 mice/litter

Irradiated (I, II):

2.6 R: 6.05 mice/litter

6 R: 5.44 mice/litter

STUDIES IN THE COMPARATIVE PHYSIOLOGY AND ANATOMY OF RADIOSENSITIVITY AND AGING

George A. Sacher, Everett F. Staffeldt and David L. Ford

PURPOSE AND METHODS

The purpose is to seek a basis for the prediction of radiation life shortening in man in terms of data on laboratory animals. Previous work on this problem has shown that the longevity of a great variety of mammalian species—including man—can be estimated accurately as a function of the adult body and brain weights. Since longevity depends on both brain size

TABLE 10. EXPERIMENTAL PLAN

Subgroup	Irradiation period	Daily doses, R/day
I	Conception to birth	2.6 and 6
II	Conception to death*	2.6 and 6
III	Birth to death*	2.6 and 6
IV	100 days of age to death*	2.6 and 6
V	Unirradiated control	—

* Irradiation for the duration of life.

The data on percent survival to weaning (4–5 weeks of age) are:

Unirradiated (IV, V): 80.9%

Irradiated:	2.6 R/day	6 R/day
Group I	73.1%	63.1%
Group II	65.1%	55.4%
Group III	62.1%	62.3%

Higher dose levels would undoubtedly cause a sharp reduction in survival at birth and weaning. The data suggest that the exposure period between birth and weaning is at least as damaging as prenatal irradiation only.

Seven animals with congenital malformations were observed; 1 in 689 (0.15%) among the non-prenatally exposed and 6 in 472 (1.27%) among groups I and II. The malformations, however, were all typical of those noted spontaneously in the C57BL line—microphthalmia, microcephaly and agnathia.

Subsequent progress reports will examine the age-specific mortality rates from birth to death and cause-of-death data.

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and body size, it can be conjectured that the radiation life shortening for a species will also depend on these two quantities.

This project will 1) investigate the differences in aging processes between long-lived and short-lived species, where metabolic rate and degree of cephalization can separately be held constant, and 2) determine the extent to which these differences are associated with

different susceptibility to radiation life shortening. A group of small rodent species (20–200 g body-weight range) will be used, since they provide an adequate range of independent variation of the two size parameters and yet permit the experiment to be carried out with minimum expenditure of time and money.

The procedures to be used are 1) determination of survival curves and disease patterns on several dozens of each species, 2) longitudinal study of age changes in vital physiological and morphological characters, 3) characterization of the several species with respect to precision and adaptability of physiological regulations and other psychophysiological performances, and 4) measurement of dosage-survival relations for conditions of periodic exposure to gamma rays and fast neutrons.

PROGRESS REPORT

During the past year, we have continued our program of obtaining information on the management of wild rodents under laboratory conditions. We have been generally successful in maintaining all the species we have tested. Where breeding has not been a success we have been able to trap sufficient numbers of animals for our purposes. We now have established healthy breeding colonies of *Peromyscus leucopus*, *Sigmodon hispidus*, *Mastomys natalensis*, *Mus musculus*, and to a lesser degree, *Neotoma albigula*. These animals are providing us with such information as longevity records, breeding records, pathology, histology, measurement of cell turnover by thymidine labelling, acute radiation responses, and radiation life-shortening effects. In the future, we will include the measurement of psychological capabilities.

In the past year, we have been able to acquire small samples of an additional twenty-five species of wild rodents. Eight of these new additions are species of *Peromyscus* which will provide us with a good sample cross section for the whole genus. Other new acquisitions

include such desert species as the round-tailed ground squirrel, pocket mouse, banner-tail kangaroo rat and cactus mouse. Because of the strict nature of the breeding requirements for these species, they have been used for anatomical measurements only. All new species are sampled for parasites, pathology, and routine histology.

Our tree squirrel population includes the flying squirrel, tropical red squirrel as well as grey and fox squirrels.

In the last six months we have begun studies of the life-shortening effects of low-dose daily gamma irradiation with *Tamias striatus*, *Mastomys*, and *Peromyscus leucopus*. Groups of animals will be exposed to levels of gamma irradiation from 12 R/d to 125 R/d for life. As more space becomes available *Mus musculus* and *Sigmodon hispidus* will be added to this group. Early breeding success with a new species, *Peromyscus californicus*, indicates it, too, may fit into this program.

In the coming year we will continue to acquire small numbers of various rodent species to add to our survey of the comparative anatomy and physiology of the Rodentia. This work will provide a foundation for the more intensive study of the aging process, physiology and radiation responses of six species of myomorph and sciurormorph rodents. These are: *Tamias*, *Sigmodon*, *Mastomys*, *P. leucopus*, *P. californicus* and *Mus musculus* (wild type). All except *Tamias* (chipmunk) breed in the laboratory, and the group gives a fair range of body and brain size as well as a considerable range of longevities (from about 3 to about 8 years). It does not appear likely that the comparative study will include a hystricomorph species, since the guinea pig is not suitable and no other species of smaller size is available.

A report on the sensitivity of several wild species to acute radiation lethality appears separately (Vogel and Sacher).

ACUTE GAMMA-RADIATION MORTALITY IN FIVE SPECIES OF WILD RODENT

Howard H. Vogel, Jr. and George A. Sacher

PURPOSE AND METHODS

Several species of wild rodent have been trapped and maintained in the animal colony over the past year. It is planned to use some of these species in comparative studies in radiation, longevity, and behavior. The

following preliminary exposures to gamma rays were carried out to establish acute radiation responses. All exposures were carried out in the high-level gamma room for 20 minutes. The rodents were irradiated in plastic ice-cream cartons developed for the Janus program.

PROGRESS REPORT (Tables 11 and 12)

TABLE 11. RADIATION RESPONSE OF *Mastomys*,
THE MULTIMAMMATE MOUSE*

Dose, R	Females	Males	Total number	% mortality (30 days)
700	0/2†	0/2	0/4	0
900	0/2	0/2	0/4	0
1100	7/13	6/14	13/27	48.2
1175	12/12	9/11	21/23	91.3
1250	11/11	6/12	17/23	73.9
1300	2/2	2/2	4/4	100.0

* LD_{50/30} : 1043.9 ± 57.4 R

m: 0.004 ± 0.002

Total number irradiated: 85

Mean survival time of 55 decedents: 11.5 days.

† Number dead in acute period/number irradiated.

Under similar irradiation conditions, the LD_{50/30} for the CF No. 1 mouse was approximately 930 R.

The thirteen-lined ground squirrel (*Citellus tri-decemlineatus*) survived 900 R, showed 85% mortality in 30 days after 1200 R and 100% after 1400 R.⁽¹⁾

TABLE 12. COMPARATIVE RADIATION RESPONSE TO
GAMMA RAYS OF THREE WILD RODENTS,
BOTH SEXES

	<i>Mus-musculus</i> (house mouse)	<i>Sigmodon hispidus</i> (cotton rat)	<i>Peromyscus leucopus</i> (deer mouse)
Dose, R			
700	0/5*	0/3	1/4
800			0/2
900	0/5	0/3	0/6
1100	5/5	2/3	4/8
1250			3/4
1300	5/5	2/3	2/4
1500	4/4	3/3	4/4
Total dead/total number irradiated	14/24	7/15	14/32
Mean survival time of 30-day decedents, days	11.6	8.3	7.5

* Number dead/number irradiated.

These figures were obtained when the animals were kept at approximately 23°C.

REFERENCE

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RADIATION AND BEHAVIOR: RUNNING ACTIVITY IN PEROMYSCUS LEUCOPUS
BEFORE AND AFTER EXPOSURE TO 100 RADS FISSION NEUTRONS

Sarah Williams* and Howard H. Vogel, Jr.

PURPOSE AND METHODS

The purpose of this study was to observe the effects of a single acute exposure to fission neutrons on running activity in a deer mouse (*Peromyscus leucopus*).

Deer mice were placed in an activity cage, 14½ × 14½ × 9½ inches, with a circular raceway around which six infrared beams were symmetrically located at 60° intervals. As the animal moved around the runway, these beams were broken and a counter operated by the electrical impulses generated identified the rate of activity.

Mice were placed singly in this cage, two or three times a day, and were run for 5-minute intervals. Six female adult *Peromyscus leucopus* were observed for 6 days prior to irradiation and for 14 days following exposure to neutrons. The 10-minute, whole-body exposure to fission neutrons was carried out at the CP-5 reactor. The animals were isolated in a temperature-

controlled room (23°C); lights were on in this room for 24 hours.

PROGRESS REPORT (Table 13)

TABLE 13. ACTIVITY (RUNNING) OF *Peromyscus leucopus*
PRIOR TO AND AFTER EXPOSURE TO 100 RADS OF
WHOLE-BODY, FISSION NEUTRON RADIATION

Mouse No.	Daily running activity	
	Before irradiation, c/5 min (Mean values, 6 days)*	After neutron exposure, c/5 min (Mean values, 14 days)*
1	162.3 ± 23.9	143.1 ± 27.8
2	118.5 ± 19.7	45.1 ± 5.4
3	151.7 ± 15.8	57.6 ± 5.4
4	91.0 ± 13.5	41.2 ± 5.2
5	129.3 ± 11.3	72.9 ± 11.1
6	174.8 ± 24.9	57.1 ± 6.3

* University of Kansas.

* c = counts

CONCLUSIONS

1. Although there was marked individual variation, there was significant reduction in running activity (usually in excess of 50% preirradiation values) in five of the six mice exposed to 100 rads of fission neutrons.

2. An evident preirradiation estrous cycle, with increased activity every 4 days, was not clearly observed after irradiation.

3. There appeared to be a significant difference in the running activity of different generations (P, F₁, and F₂) of *Peromyscus* in this activity cage.

EFFECTS OF LIFETIME PERIODIC GAMMA-RAY EXPOSURE ON THE SURVIVAL AND PATHOLOGY OF GUINEA PIGS

John H. Rust, Robert J. Robertson, Everett F. Staffeldt, George A. Sacher, Douglas Grahn, and R. J. Michael Fry*

This report is being made because there is little information on the pathological changes in guinea pigs exposed to lifetime irradiation.⁽¹⁾ Despite the fact that the experiment was designed to study the life-span of the chronically irradiated animals,⁽²⁾ and, therefore, no sacrifice series was undertaken, the information obtained about the pathological changes was of sufficient interest to warrant this report.

PROGRESS REPORT

The number of animals and the experimental design are shown in Table 14. The guinea pigs were obtained from Tumble Brook Farm. The selection of tissues at necropsy was determined by the degree of autolysis present and the discretion of the prosector. Because of the long life-span of the guinea pig, the experiment has lasted for more than 8 years and several different prosectors have conducted the necropsies. All these factors lead to a lack of consistency which is regrettable but unavoidable. The incidences of the lesions given in Tables 16-18 are divided into those based on diagnosis made from the gross appearances and those made on microscopic examination. The diagnosis by gross inspection was made in animals where tissues were not saved because of the degree of autolysis but in which the appearances were reasonably characteristic for a presumptive diagnosis to be made.

The incidences of cirrhosis, fatty liver, pulmonary tumors, lymphosarcoma, and chronic kidney disease are given in Tables 15-18. A later report will discuss the incidence of the different types of lymphosarcoma, pulmonary tumors, and chronic kidney disease that were found.

CONCLUSIONS

From the results presented, it appears that 1) there is no definite increase in fatty metamorphosis or cir-

rhosis in the liver due to irradiation, 2) the incidence of pulmonary tumors was low and there was no increase due to irradiation, 3) the number of cases of lymphosarcoma confirmed by microscopical examination was small but the results suggest that there may have been an increase in the irradiated animals, 4) there was no clear-cut increase in the incidence of chronic kidney disease due to irradiation.

The mean after-survival times (MAS) of the treated groups and controls are shown in Figure 20, plotted as logarithm of MAS vs. weekly dose in roentgens. The relationship is approximately linear and the life-short-

TABLE 14. EXPERIMENTAL DESIGN

Group	Daily exposure, R	Number of weekly exposures*	Total weekly dose, R	Number of animals in study	Number of animals not reported here
1	0	0	0	48	2
2	0	1	0	24	1
3	0	4	0	24	4
4	0	5	0	24	0
Total controls				120	7
5	1.35	1	1.35	48	1
6	1.35	1	1.35	48	0
Total groups 5 and 6				96	1
7	1.35	4	5.4	48	0
8	2.6	5	13.0	40	0
9	6.0	4	24.0	12	0
10	24.0	1	24.0	12	0
11	6.0	6	36.0	16	0

* In the case of the control animals the first group remain in the animal room, the second, third and fourth were moved from the animal room each "exposure" day and remained in the hallway outside of the exposure room in an attempt to simulate closely the movement of the exposed animals.

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TABLE 15. CIRRHOSIS AND FATTY METAMORPHOSIS OF THE LIVER

Group	Period, days										Total
	0-250	251-500	501-750	751-1000	1001-1250	1251-1500	1501-1750	1751-2000	2001-2250	2251-2500	
Controls (Groups 1, 2, 3 & 4)											
A*	47	47	45	41	31	24	17	5	2	—	47 (120)†
Cirrhosis	0	0	0	2	3	0	1	0	2		8
Fatty liver	0	2	1	3	2	2	2	1	0		13
1.35 R/wk [1.35 R 1×/wk (Groups 5 & 6)]											
A	45	42	40	37	28	19	10	5	—	—	45 (96)
Cirrhosis	1	0	3	1	1	2	2	0			10
Fatty liver	3	1	1	2	4	0	0	2			13
5.4 R/wk [1.35 R 4×/wk (Group 7)]											
A	19	19	19	19	15	10	4	1	—	—	19 (48)
Cirrhosis	0	0	0	2	0	2	0	0			4
Fatty liver	0	0	0	2	1	3	2	0			8
13 R/wk [2.6 R 5×/wk (Group 8)]											
A	25	25	24	20	16	7	2	1	—	—	25 (40)
Cirrhosis	0	0	1	1	0	2	0	0			4
Fatty liver	0	0	1	2	4	0	0	0			7
24 R/wk [6 R 4×/wk (Group 9)]											
A	6	5	3	3	—	—	—	—	—	—	6 (12)
Cirrhosis	0	0	0	0							0
Fatty liver	0	1	0	0							1
24 R/wk [24 R 1×/wk (Group 10)]											
A	4	4	3	2							4 (12)
Cirrhosis	0	0	0	0							0
Fatty liver	0	1	1	0							2
36 R/wk [6 R 6×/wk (Group 11)]											
A	6	6	5	4	2	—	—	—	—	—	6 (16)
Cirrhosis	0	0	1	1	0	—	—	—	—	—	2
Fatty liver	0	1	0	1	2	—	—	—	—	—	4

* A = Animals living at start of period.

† The number in parenthesis is the total of animals which started the experiment.

TABLE 16. PULMONARY TUMORS

Group	Periods, days										Total
	0-250	251-500	501-750	751-1000	1001-1500	1251-1500	1501-1750	1751-2000	2001-2250	2251-2500	
Controls (Groups 1, 2, 3 & 4)											
A*	91	89	82	69	55	42	25	7	1	—	91 (120)†
Microscopic diagnosis	0	0	0	0	0	0	1	1	0		2
Gross diagnosis	1	0	0	0	1	0	0	0	0		2
1.35 R/wk [1.35 R 1×/wk (Groups 5 & 6)]											
A	88	82	72	64	48	33	16	9	1	—	88 (96)
Microscopic diagnosis	0	0	0	0	0	1	0	0	0		1
Gross diagnosis	0	0	0	1	0	0	1	0	0		2
5.4 R/wk [1.35 R 4×/wk (Group 7)]											
A	38	37	36	35	29	20	8	1	—	—	38 (48)
Microscopic diagnosis	0	0	0	0	0	0	0	0			0
Gross diagnosis	0	0	0	0	0	0	0	0			0
13 R/wk [2.6 R 5×/wk (Group 8)]											
A	40	39	36	28	21	9	4	1	—	—	40 (40)
Microscopic diagnosis	0	0	0	0	0	0	0	0			0
Gross diagnosis	0	0	0	0	0	0	0	0			0
24 R/wk [6 R 4×/wk (Group 9)]											
A	12	10	7	5	—	—	—	—	—	—	12 (12)
Microscopic diagnosis	0	0	0	0							
Gross diagnosis	0	0	0	0							
24 R/wk [24 R 1×/wk (Group 10)]											
A	12	8	5	2	1	—	—	—	—	—	12 (12)
Microscopic diagnosis	0	0	0	0	0						
Gross diagnosis	0	0	0	0	0						
36 R/wk [6 R 6×/wk (Group 11)]											
A	6	6	5	4	2	—	—	—	—	—	6 (16)
Microscopic diagnosis	0	0	0	0	0						
Gross diagnosis	0	0	0	0	0						

* A = Animals living at start of period.

† The number of animals which were used in this study.

TABLE 17. LYMPHOSARCOMA

Group	Period, days										Total
	0-250	251-500	501-750	751-1000	1001-1250	1251-1500	1501-1750	1751-2000	2001-2250	2251-2500	
Control (Groups 1, 2, 3, & 4)											
A*	92	87	81	70	54	41	24	7	2	—	92 (120)†
Gross diagnosis	1	0	0	0	0	0	0	0	0		1
Microscopic diagnosis	0	0	0	2	2	0	0	0	0		4
1.35 R/wk [1.35 R 1×/wk (Groups 5 & 6)]											
A	88	82	72	64	48	33	16	9	1	—	88 (96)
Gross diagnosis	0	1	0	0	0	0	0	1	0		2
Microscopic diagnosis	0	0	1	0	2	0	0	1	0		4
5.4 R/wk [1.35 R 4×/wk (Group 7)]											
A	38	37	36	35	29	20	8	1	—	—	38 (48)
Gross diagnosis	0	0	0	1	3	1	0	0			5
Microscopic diagnosis	0	0	0	0	0	0	0	0			0
13 R/wk [2.6 R 5×/wk (Group 8)]											
A	39	38	35	27	21	9	4	1	—	—	39 (40)
Gross diagnosis	0	0	0	0	0	0	0	0			0
Microscopic diagnosis	0	0	1	1	3	0	0	1			5
24 R/wk [6 R 4×/wk (Group 9)]											
A	12	10	7	5	—	—	—	—	—	—	12 (12)
Gross diagnosis	0	0	0	0							0
Microscopic diagnosis	0	0	0	0							0
24 R/wk [24 R 1×/wk (Group 10)]											
A	12	8	5	2	1	—	—	—	—		12 (12)
Gross diagnosis	2	1	0	0	0						3
Microscopic diagnosis	0	0	0	0	0						0
36 R/wk [6 R 6×/wk (Group 11)]											
A	15	13	8	4	2	—	—	—	—	—	15 (16)
Gross diagnosis	0	1	0	1	0						2
Microscopic diagnosis	0	0	0	0	1						1

* A = Animals living at start of period.

† The number of animals which were used in this study.

TABLE 18. CHRONIC KIDNEY DISEASE

Group	Periods, days										Total
	0-250	251-500	501-750	751-1000	1001-1250	1251-1500	1501-1750	1751-2000	2001-2250	2251-2500	
Control (Groups 1, 2, 3 & 4)											
A*	93	89	82	69	55	42	25	7	2	—	93 (120)†
Gross diagnosis	0	0	1	0	2	6	2	0	0		11
Microscopic diagnosis	0	2	4	9	6	5	15	4	2		47
1.35 R/wk [1.35 R 1×/wk (Groups 5 & 6)]											
A	88	82	72	64	48	33	16	9	1	—	88 (96)
Gross diagnosis	0	2	0	1	3	4	1	0	0		11
Microscopic diagnosis	1	2	2	9	11	8	5	7	1		46
5.4 R/wk [1.35 R 4×/wk (Group 7)]											
A	38	37	36	35	29	20	8	1	—	—	38 (48)
Gross diagnosis	0	0	0	0	0	2	2	0			4
Microscopic diagnosis	0	0	0	3	3	5	4	1			16
13 R/wk [2.6 R 5×/wk (Group 8)]											
A	40	39	36	28	21	9	4	1	—	—	40 (40)
Gross diagnosis	0	0	0	1	1	0	0	0			2
Microscopic diagnosis	0	0	3	3	6	3	1	1			17
24 R/wk [6 R 4×/wk (Group 9)]											
A	12	10	7	5	—	—	—	—	—	—	12 (12)
Gross diagnosis	0	0	0	0							0
Microscopic diagnosis	1	1	0	3							5
24 R/wk [24 R 1×/wk (Group 10)]											
A	12	8	5	2	—	—	—	—	—	—	12 (12)
Gross diagnosis	0	0	0	0							0
Microscopic diagnosis	0	1	2	0							3
36 R/wk [6 R 6×/wk (Group 11)]											
A	15	13	8	4	2	—	—	—	—	—	15 (16)
Gross diagnosis	0	0	0	0	0						0
Microscopic diagnosis	0	1	1	2	1						5

* A = Animals living at start of period.

† The number of animals which were used in this study.

FIG. 20.—Relation of mean after-survival time to weekly dose for guinea pigs exposed to gamma rays periodically throughout adult life. Circles are for pigs exposed 4 to 6 times weekly (see Table 14). Dot is for group exposed once per week. Triangles are for a repeat study at 0 and 5.2 R/week and are median rather than mean survival times. Crosses are data of Lorenz, *et al.*⁽¹⁾

ening coefficient is about 0.06 days/R. The data of Lorenz, *et al.*⁽¹⁾ are plotted in Figure 20 for comparison, and are in close agreement with ours.

The absence of any life shortening at 5.4 R/week in the present study is undoubtedly a significant departure from the trend at higher dose levels. However, a repeat of the comparison between 0 and 5.2 R/week, using a total of 48 pigs, is now under way, and shows about the expected shortening of life (Figure 20).

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EFFECTS OF IONIZING RADIATIONS ON DURATION OF LIFE IN THE CHICKEN

S. Phyllis Stearner and Emily J. B. Christian

PURPOSE AND METHODS

To determine long-term radiation effects on survival time, white leghorn roosters, irradiated at 3 to 4 days after hatching, are being maintained for duration of life. An important part of this investigation involves observations of control birds because no long-term study of chickens maintained under laboratory conditions is known. The radiation exposures were either to 200 kv x-rays or to Co⁶⁰ gamma rays. All x-ray exposures were delivered in 30 minutes. Gamma-ray exposures were given in 6 to 60 minutes or in 24 hours in order to investigate the effect of exposure time on dose effectiveness. In most instances doses covered the 30-day lethal range. Survivors at 30 days were caged individually and are being observed regularly.

PROGRESS REPORT

The control mortality reached 50% in about 2000 days. Only part of the control group has been followed for a longer period; of 40 birds started more than 9 years ago, survival is about 10%.

n gamma-irradiated birds, exposed in 6 to 60

minutes, the mortality rate was higher than in the controls. Of those alive at 30 days after exposure to doses in the 30-day lethal range, about 50% had survived at the end of 1000 days, and there was less than 10% survival at about 2000 days. There appears to be no correlation between dose and survival time. The insult sustained in chicks surviving the acute period appears to be about the same when measured by long-term survival or life-span. Doses protracted over 24 hours were less effective in reducing survival time, but again doses in the 30-day lethal range were comparable in their effect on mortality rate. We have also observed a lack of relation between dose and growth effect for doses within this range. X-irradiated roosters (dose of 1000 to 1200 R) showed an unusually high mortality during the first year (based on those alive at 30 days). Deaths in this period were frequently associated with severe liver atrophy. Between 1 and 5 years, however, the survival curve paralleled that of the control, but mortality was high between 6 and 7 years, and only the last decile survived more than 7 years.

INFLUENCE OF LOW AND HIGH RATE OF EXPOSURE ON THE WEIGHT OF THE CHICKEN

Sylvanus A. Tyler and S. Phyllis Stearner

PURPOSE AND METHODS

One of the consequences of exposure to ionizing radiation is an alteration in magnitude and pattern of subsequent growth. In this study, a quantitative description of the dependence of weight on amount and degree of protraction of the radiation is attempted. To describe the change in weight due to exposure, the radiation insult sustained must be severe enough to detect differences in weight despite an inherently large, animal-animal variation. Thus, young chicks that survived for 30 days after receiving an acute dose of x-rays or Co⁶⁰ gamma rays were selected as the basic population for study.

Investigation of acute lethal injury in the chick⁽¹⁾ has shown that, if 30-day mortality is used as a criterion, the effectiveness of a single dose decreases as the rate at which the dose is delivered diminishes. A proposed explanation for this observation is that recovery processes operate during radiation exposure to reduce the accumulation of lethal injury. Survival of the animal requires that the net injury leading to death remain below a critical magnitude. Although an irradiated animal survives, it has suffered an insult which may or may not be "recoverable," even in part, if other criteria of radiation damage sustained (such

as the effect on growth) are used. The object of this investigation is to determine if a recovery can be demonstrated using body weight changes as the indicator.

The analysis of differences in patterns of growth relative to radiation dose and exposure time is expedited by assuming that the age-growth relationship is comprised of two age-contiguous parts:

1. an initial growth phase that is arbitrarily limited to ages less than 170 days; and
2. a phase of relatively constant growth rate that roughly extends over ages from 170 to 800 days.

This phase of growth will not be discussed.

Male, white leghorn chicks at 3 to 4 days after hatching received single total-body exposures of either 250 kv x-rays or Co⁶⁰ gamma rays. The x-ray dose was delivered in approximately 35 minutes and the γ -ray dose was either given in 60 minutes or less (fast rate) or protracted over 24 hours (slow rate). Between 31 and 41 days after the beginning of the exposure (i.e., 34 to 45 days of age), surviving birds were caged individually and maintained under conditions standard for our animal quarters. Weights were recorded regularly for the duration of life and those at ages less

FIG. 21.—Mean weight versus age of 30-day survivors among chicks exposed to acute doses of gamma rays at fast rate. Controls, ●; 700 R, ○; 1100 R, ×.

FIG. 22.—Mean weight versus age of 30-day survivors among chicks exposed to acute doses of gamma rays at slow rate. Controls, ●; 700 R, +; 900 R, ×; 1200 R, ○; 1600 R, ◆; 2000 R, ◇.

FIG. 23.—Relation between the estimated weight at start of irradiation and dose. Slopes of the unweighted least squares lines: gamma ray (fast rate) = -0.0192 ± 0.0014 ; gamma ray (slow rate) = -0.0165 ± 0.0013 .

than 800 days are used in the analysis (see Figures 21 and 22).

PROGRESS REPORT

For the initial growth phase, the relationship between weight, $W(t)$, and age, t , is mathematically described by the Gompertz function,⁽²⁾

$$W(t) = W_0 \text{Exp} \left\{ \frac{A_0}{\alpha} [1 - \text{Exp}(-\alpha t)] \right\} \quad (1)$$

with

$$A_t = A_0 \text{Exp}(-\alpha t). \quad (2)$$

To determine the constants A_0 and α , the linear regression model (3) was used.

$$\ln \left\{ \frac{1}{W(\xi_i)} \left[\frac{\Delta W_i}{\Delta t_i} \right] \right\} = \ln A_0 - \alpha(\xi_i), \quad (3)$$

where $\Delta t_i = t_{i+1} - t_i$ and $\Delta W_i = W_{i+1} - W_i$ represent the differences between consecutive ages and corresponding weights, the value ξ_i and $W(\xi_i)$ are taken as

$$\xi_i = t_i + \frac{2}{3} \Delta t_i \quad \text{and} \quad W(\xi_i) = W_i + \frac{2}{3} \Delta W_i, \quad (4)$$

and i denotes the ordinality of t . Estimates of the constants A_0 and α were not significantly different between dose groups, and, thus, all groups were combined and the resulting estimates, $A_0 = 0.0888$ and $\alpha = 0.02077$, were accepted as constants of the whole experience. This leads to the postulation that the effect of dose on

weight reduces to a relationship between W_0 and dose. In Figure 23, this relationship is shown through the use of $W_{3.5}$, in place of W_0 , the weight at the average age of the chick when exposure began. The theoretical curves drawn in Figures 21 and 22 are based on the accepted constants A_0 and α , and the $W_{3.5}$ corresponding to the dose group designated.

CONCLUSIONS

From the figures we observe that for the initial growth phase,

1. the relationship between $W_{3.5}$ and dose for slow- and fast-rate gamma rays is approximately linear with similar slopes over the range of doses covered;

2. the three x-ray points are not appreciably different from the fast-rate gamma-ray expectations;

3. radiation dose delivered at a fast rate is more effective than the same dose delivered at a slow rate; and

4. the reduction in effectiveness for a 24-hour exposure is roughly 400 R.

The difference between acute LD_{50} doses for a 60-minute and 24-hour exposure is about 400 R.

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RELATION OF DOSE AND EXPOSURE TIME FOR ACUTE RADIATION MORTALITY IN THE ADULT CHICKEN

S. Phyllis Stearner, Margaret H. Sanderson, and Emily J. B. Christian

PURPOSE AND METHODS

The kinetics of injury accumulation during irradiation, for early mortality (0 to 2 days after exposure), has been described in the young chick and chick embryo.¹⁻³ The empirical model, developed to describe the course of mortality in terms of dose and exposure time, postulated a constant linear reversal of dose effectiveness during irradiation. For single exposure periods up to 24 hours, the data revealed no departure from a linear relation. It has been assumed, therefore, that only a time-dependent effect is involved in the early mortality and that the loss of dose effectiveness during exposure occurs at a constant rate that is independent of the delivered-dose rate. Our observations have now been extended to include the six-month rooster so that we can compare the kinetics of injury

accumulation in the adult chicken with that of the young chick and the chick embryo. This comparison is of special interest because it was established previously that the early mode of death is comparable in the chick embryo, the 3-day-old chick, and the adult rooster.^(2, 4, 5)

White leghorn cockerels, received 2 days after hatching, were reared to 6 months of age in our animal facilities. At 30 days of age, the birds were wing-clipped and transferred from brooders, 1 or 2 to a cage, until the experiment was completed. The roosters were irradiated in perforated aluminum boxes that were arranged radially around the Co^{60} source, and 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 pro-

tracted over 6 different time periods ranging from 30 minutes to 1 day. The delivered dose ranged from 775 R to 15,500 R to span the lethal range for the exposure times that were employed. There were approximately 25 animals in each dose-time group, and 4 to 6 dose groups at each exposure time. More than 850 birds are represented in the analysis reported here. This report is concerned with mortality within 2 days after the beginning of the exposure although all groups were observed for 30 days. Mortality was recorded daily; survivors after 30 days were sacrificed and necropsied for observations of gross lesions.

PROGRESS REPORT

The dose-mortality relation for Co^{60} γ irradiation delivered in 30 minutes is shown in Figure 24. With an increase in the exposure time, there is a constant decrease in the dose effectiveness, as illustrated by an

FIG. 24.—Dose mortality relation for 30-min exposures to Co^{60} γ rays (probit transform). Constants of the least-squares line are $\text{LD}_{50} = 1037 \pm 8$ R, slope = 0.006360 ± 0.000355 .

TABLE 19. CONSTANTS OF THE LEAST-SQUARES PROBIT LINES FOR 0- TO 2-DAY MORTALITY vs. DOSE

Exposure time, min	LD_{50} , R	Standard error LD_{50} , R	Slope	Standard error slope	σ
30	1037	8	0.00636	0.00036	157
90	1435	53	0.00275	0.00057	364
240	2853	115	0.00126	0.00030	794
480	5562	107	0.00077	0.00010	1296
960	9909	229	0.00038	0.00006	2614
1440	13533	253	0.00033	0.00004	3045

TABLE 20. MEAN D/D_{30} RATIOS WITH RESPECT TO EXPOSURE TIME

Exposure time, min	Groups	Mean D/D_{30}	Standard deviation
90	6	1.365	0.145
240	6	2.722	0.247
480	6	5.206	0.339
960	6	9.114	0.767
1440	4	12.648	0.902

FIG. 25.—Relation between mean D/D_{30} and exposure time. Constants of the least-squares line, forced through 1.0 at $t = 30$ min.; slope = 0.008458 ± 0.000161 ; $1/b = 118.2$ min., $S_{1/b} = 1.2$ min.

increase in LD_{50} values (Table 19). Other constants of the least square lines (probit transforms) are also presented. The distribution of mortality, with respect to dose for groups exposed for 30 minutes, was used as a basis for deducing the kinetic constants of the time-dependent effects with which the early mortality is associated. The D/D_{30} ratios for all exposure-time groups were computed; the mean ratios for each exposure time are shown in Table 20. The least-squares line of mean D/D_{30} ratios vs. the exposure time, with the restriction that $D/D_{30} = 1.000$ at $t = 30$ minutes, is plotted in Figure 25.

The linear model of reversible effects that has been assumed⁽¹⁾ is expressed by the formula

$$\frac{D}{D_0} = 1 + \frac{t}{\tau}$$

where D/D_0 is the ratio for a protracted exposure of t minutes and D_0 is the instantaneous dose that produces the same mortality as D . The slope is defined as the reciprocal of the time constant τ . From D/D_{30} values, the estimate of D/D_0 was computed. An estimate of τ was determined from the slope of the fitted D/D_{30} line [$\tau = (1/b) - 30$, where b is the slope]. From these data, the estimate of τ was found to be 88.2 ± 1.2 minutes. This constant was used to construct the estimated D/D_0 line (Figure 25) and to determine the estimated instantaneous dose LD_{50} of 773.4 R. An estimate of reversal or repair rate in terms of dose can be obtained from the relation between the LD_{50} values for D_0 and the statistical estimate of τ . From this calculation it is found that 50% of the animals can reverse or repair at a rate equal to or greater than 8.76 R/min, those time-dependent effects that are capable of producing death within the first 48 hours after exposure. On the basis of a linear reversal, the repair rate is assumed to be independent of accumulated dose, and is instead, an inherent characteristic of the individual animal.

CONCLUSION

The time constant associated with the time-dependent process for early (0-2 days) mortality in the rooster is less than that previously reported for the young chick and for the chick embryo. In the 3-day chick, the value of τ for the first-period lethal effect is 146 ± 3 minutes, while for the 6-day chick embryo the corresponding value is 136 ± 3 minutes. We believe, however, that the value for τ , comparable to that reported for the chick and chick embryo, is more

closely approximated by the reciprocal of the slope of the D/D_{30} line, that is, $1/b$ or 118 minutes. In both the chick and chick embryo a lag of about 60 minutes was observed for D/D_0 ratios when D_0 was represented by groups exposed for less than 10 minutes.^(1,3) Data from the rooster do not include a sufficient number of shorter exposure periods to identify the presence of a lag.

Survival studies now in progress on the rooster include the 3- to 30-day lethal range. In addition to an analysis of the effects of protraction on radiation effectiveness, experiments involving split exposures are planned to determine whether an "over-recovery" such as has been described in the young chick can also be demonstrated in the adult.⁽⁴⁾

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EVIDENCE OF "OVER-RECOVERY" FOLLOWING EXPOSURE TO IONIZING RADIATIONS

S. Phyllis Stearner, Sylvanus A. Tyler, and Margaret H. Sanderson

PURPOSE AND METHODS

In a previous report, the dependence of initial acute mortality (0 to 2 days after irradiation) in the young chick on dose and length of interval between the 2 fractions of a split exposure was described. Results revealed that in addition to recovery processes an induced resistance contributes to the reduction in efficiency of the delivered dose.⁽¹⁾ This resistance or "over-recovery" can be thought of as an increase in the basic tolerance of the animal to radiation. Investigations reported here involve the effects of split doses on acute mortality within 3 to 30 days after exposure of young chicks. Similar experiments now in progress are directed toward analysis of mechanisms

of recovery and possible resistance associated with split exposures in the mouse; results will be presented in a later report.

Three-day-old white leghorn cockerels were exposed to Co^{60} gamma rays. Radiation doses were within the 30-day lethal range and were delivered as single or split (2-dose) exposures. Exposure time for the fast single dose was 30 minutes. Each fraction of the split exposures was delivered in 15 minutes, separated by intervals varying from 2 to 24 hours. In the split-dose groups, the conditioning dose was either 300 or 700 followed after a predetermined interval by a challenging dose. For each first dose and interval, 4 to 8 challenging doses were selected to span the lethal range

FIG. 26.—Dose-mortality distribution for deaths in the period 3–30 days after 30-minute exposures to CO^{60} γ rays. Data are fitted to a cumulative normal curve with $\text{LD}_{50} = 957 \pm 12$ R and $\sigma = 173$ R.

for deaths within 3 to 30 days. Single exposures were protracted over 3 and 4 hours. The number of chicks in each dose-time group ranged from 40 to 60, and a total of more than 4800 chicks was used. The proportion of the animals dying within the period 3 to 30 days after the conditioning exposure, of those alive at the beginning of the period, was used to measure the effectiveness of the irradiation.

PROGRESS REPORT

Radiosensitivity of the 3-day chick to single exposures of Co^{60} gamma rays, delivered in 30 minutes, is shown in Figure 26. To demonstrate the variation in radiation effect as a function of time after the conditioning exposure, sensitivity in split-dose groups was compared with that in groups that received single 30-minute exposures. For each split-dose group, the ratio of total dose ($D_1 + D_2$) delivered in two exposures to the single dose (D_0) required to produce the same mortality was computed.* Mean ratios for groups exposed at each interval in the 300- and 700-R series are shown in Table 21. The relation between mean ($D_1 + D_2$)/ D_0 ratios and time is shown in Figure 27. The theoretical line for comparable results with single protracted exposures is drawn, using constants estimated in previous experiments.⁽²⁾

At two hours after a conditioning dose of 300 R, the amount of dose nullified (in terms of roentgens) is

* The following notations are used: D = total delivered dose, either as a single or split exposure; D_1 = first or conditioning dose; D_2 = second or challenging dose; D_0 = single 30-min exposure; $\Delta D = (D_1 + D_2) - D_0$.

TABLE 21. MEAN $(D_1 + D_2)/D_0$ RATIOS AND ABSOLUTE AMOUNT OF DOSE REDUCTION, WITH RESPECT TO TIME BETWEEN EXPOSURES

D_1 , R	Interval, hrs	Number of groups	Mean ratio	Standard deviation	Total* dose, R	ΔD , R	Resistance,† R
—	0	6	1.000		957	—	
300	2	4	1.375	0.066	1316	359	59
	3	4	1.437	0.016	1375	418	113
	4	5	1.506	0.077	1441	484	184
	6	6	1.435	0.094	1373	416	116
	8	6	1.361	0.040	1302	345	45
	16	5	1.331	0.117	1274	317	17
	24	4	1.411	0.038	1350	393	93
700	2	6	1.495	0.103	1431	474	174
	3	7	1.537	0.105	1471	514	214
	4	8	1.525	0.034	1459	502	202
	6	8	1.471	0.094	1413	456	156
	8	7	1.419	0.081	1358	401	101
	16	6	1.360	0.077	1302	345	45
	24	6	1.415	0.051	1354	397	97

* Total dose equals mean ratio times 957 R.

† Dose reduction in excess of 300 R.

approximately equal to that reduction experienced when an exposure is protracted over an equivalent time period. With longer intervals there is little additional reduction in dose sensitivity. The mean $(D_1 + D_2)/D_0$ ratios reach a maximum at 3 to 4 hours. A nonrandom fluctuation in sensitivity to a second exposure is suggested by the pattern of ratios for intervals between 4 and 24 hours. Resistance to the second dose is at a minimum at about 16 hours. Differences between mean ratios for intervals from 4 to 24 hours are greater than can be statistically accounted for by random variation (based on the 0.05 level of Snedecor's F statistic as determined by a one-way analysis of variance). After a conditioning exposure of 700 R, reduction in dose effectiveness was slightly, but significantly, greater than after 300 R (Figure 27). The $(D_1 + D_2)/D_0$ ratio reached a maximum after 3 to 4 hours, and subsequently decreased to a minimum at approximately 16 hours. A nonrandom variation in these ratios over intervals from 4 to 24 hours was found.

Complete recovery from a conditioning dose of 300 R is represented by a $(D_1 + D_2)/D_0$ ratio of 1.313 (Figure 27), based on the computed D_0 equal to 957 R for 50% mortality; in the series that received this conditioning dose, however, all experimental ratios are above this level. Even complete recovery of the 300 R cannot account for the magnitude of the ratios found. The sensitivity ratio of pre-irradiated animals can reach these observed levels only if an additional mech-

FIG. 27.—Relation between mean D/D_0 ratios and time between exposures. ●, $D_1 = 300$ R; ○, $D_1 = 700$ R; ×, single protracted exposures. Solid line represents dose reduction for continuous exposures based on previously estimated constants.⁽²⁾

anism operates to reduce radiosensitivity. Contrary to this, however, a ratio of 1.731 represents complete recovery of a conditioning dose of 700 R, but the maximum observed ratio is 1.537. Results in the 300 R series clearly show that reduction in dose effectiveness is due to more than *simple recovery* of induced injury and an induced resistance is implied. We believe that a resistance may also be present after a conditioning dose of 700 R. If recovery of a first dose of 300 R is complete, the amount of recovery after a first dose of 700 R is approximately the same, then the additional decrease in sensitivity observed in intervals of 8 hours or less is functionally related to the size of the first dose (Table 21). Furthermore, at an interval of 16 hours the mean ratios for the two series differ only slightly. At this interval radiation sensitivity is approximately the same as if recovery of a 300 R conditioning dose were the sole effect.

CONCLUSIONS

These observations suggest:

1. resistance reaches a maximum between 3 and 4 hours and has essentially vanished by 16 hours;
2. recovery of radiation effects is time limited, for at 16 hours only a 300 R reduction in effective dose is evident, even in the 700 R group.

Actually, the time span over which recovery takes place may be less than 2 hours in the chick. The presence of a time-limited recovery process was inferred previously⁽³⁾ from observations on the mouse; in this animal the time span appeared to be about 5 hours. It is recognized that the influence of recovery and resistance on the radiosensitive status of an organism cannot be separated using mortality data alone. Al-

though previously irradiated animals may display greater radioresistance when tested by a second exposure than those that have not received a conditioning dose, the magnitude of the recovery may not be as large as we have assumed. If this is true, the resistance would comprise an even larger portion of the observed decrease in sensitivity.

It is common practice among investigators of split-dose effects in mice to describe observed changes in sensitivity in terms of "roentgens repaired." Such a measure is inadequate since processes other than repair are repeatedly demonstrated. In the mouse, cyclic fluctuations in sensitivity to 30-day mortality are evident with intervals up to 1 to 2 days.⁽⁴⁾ In addition, evidence of nonrandom fluctuations in cell sensitivity during intervals up to 24 hours are available from studies of mammalian cells in culture.⁽⁵⁻⁷⁾ Such shifts, acting in addition to recovery processes, could increase radiation tolerance to levels exceeding that of unirradiated animals. In the mouse, changes in sensitivity during this period tend to be smaller than those in the chick and have not been reported to exceed the amount of the first dose. In the chick, however, existence of a resistance to mechanisms governing 3-to-30-day mortality can be recognized with certainty.

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INVESTIGATIONS INTO MECHANISMS OF EARLY RADIATION LETHALITY AND RESISTANCE IN THE YOUNG CHICK AND CHICK EMBRYO

S. Phyllis Stearner, Arthur K. Katoh, Emily J. B. Christian, and Margaret H. Sanderson

PURPOSE AND METHODS

Characteristic features of the initial radiation syndrome in the young chick and chick embryo indicate that circulatory collapse plays a dominant role in mortality. It is believed that underlying mechanisms may involve alterations in permeability, especially of the capillary endothelium. In the young chick, irradiation is followed by rapid decrease in plasma volume and in plasma albumin concentration and by lesser effects in the plasma globulin fractions. The rate of decrease in the plasma protein concentration is more rapid after a lethal (1400 R Co^{60} γ rays in 10 minutes) than after a minimal or sublethal irradiation (700 R in 10 minutes); it has been assumed that it is not the low level of albumin concentration that is significant for early mortality but the rate at which the hypoproteinemia develops.⁽¹⁾ If the rate of loss is gradual, as it is after exposure to 700 R, the animals are able to compensate for alterations in volume and composition of the plasma; after 24 hours, normal levels are approached. The permeability effect is exposure-time dependent, because when exposures are protracted over longer periods (up to 24 hours) much larger doses are required to produce equivalent effects.

The relation of permeability changes to the increase in radiation tolerance that develops within a few hours after an initial conditioning exposure is important. Some years ago, we demonstrated that the initial acute mortality (0 to 2 days after exposure) in the 3-day chick is dependent on the dose and on the duration of the interval between split exposures, and that in addition to these recovery processes there is also evidence of an induced resistance that reduces the magnitude of injury resulting from a second exposure. For example, a first dose of 700 R followed, after a 4-hour interval, by a second dose of 1400 R resulted in only about 10% mortality in 24 hours. This is comparable

to a lethal effect of 700 R alone while more than 90% mortality is expected within 5 hours after exposure to a single dose of 1400 R.

PROGRESS REPORT

Investigations of the circulatory changes that occur after split exposures indicate that the chick is able to compensate for or to nullify effects of a second exposure. There are relatively small changes in the hematocrit within 4 hours after 700 R; if a second dose is administered at this time, only a transient increase is observed and return to near normal levels occurs rapidly. The plasma volume does not reach the low levels observed after 1400 R. The concentration of plasma proteins shows little additional change after the second exposure that could be attributed to the single dose.

Studies of the fine structure of the capillary endothelium (mesentery) in the chick has failed to demonstrate lesions in the cell membrane during periods of increased plasma protein loss following irradiation. We have not yet determined how the protein loss occurs and whether areas of localized injury are involved. In the chick embryo, evidence of circulatory collapse is associated with early radiation mortality as it is in the young chick. Increased hematocrit values in lethally-irradiated embryos indicated rapid loss of plasma volume, but there was little change in the protein concentration. After smaller radiation doses (nonlethal doses), however, preferential loss of the albumin fraction occurred and a relatively small increase in hematocrit indicated minor change in plasma volume (carried out by Steven Azuma, ACM Semester Student). In lethally-irradiated chick embryos, ultrastructural breaks in the capillary endothelial cell membrane indicate the mechanism by means of which rapid loss of plasma volume can occur.⁽²⁾ We do not yet have information concerning ultrastructural

changes in the capillary endothelium in chick embryos exposed to minimum or sublethal radiation doses, where selective loss of plasma albumin occurs. In addition, it has been demonstrated that such doses can, as they do in the young chick, result in increased tolerance to a subsequent radiation exposure. Investigations into the mechanisms of the increased tolerance will continue.

Circulatory changes in the 72-hour chick embryo are being studied microcinematographically in order to determine the nature of changes in the circulatory efficiency. Improved methods of explanting embryos of this age and of irradiating the explants have been developed. Preliminary observations suggest that lethal irradiation is rapidly followed by hemoconcentration. In localized areas, the circulating cells tend to adhere to one another and to the capillary endo-

thelium, and clumps are frequently observed. As a later change, evidence of breaks in the capillary endothelium are apparent. If circulating cells briefly enter into the breaks, they are either trapped or subsequently caught up in the stream of the circulation. Hemoconcentration and increased stickiness of the cells leads to aggregates which may plug the capillary circulation in large areas and ultimately result in complete stasis of the circulating cell mass.

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ACUTE RADIATION RESPONSE OF CF NO. 1/ANL MICE TO Co^{60} GAMMA RAYS

Howard H. Vogel, Jr., Nicholas Bink, and Richard R. Barhorst

PURPOSE AND METHODS

In preparation for the Janus neutron toxicity program, it is important to test the replicability of the acute radiation response in the CF No. 1/Anl mouse. We reported the $\text{LD}_{50/30}$ of the female CF No. 1 mouse to be 927 ± 5 R.⁽¹⁾ We decided, this year, to investigate the acute radiation response of both sexes of this strain of mouse, bred at Argonne National Laboratory. In addition, we evaluated the influence of several environmental factors on this response. All animals were irradiated in the High-Level Gamma Room for twenty minutes with whole-body doses ranging from 780 to 1050 R. All mice were exposed singly, in plastic cartons developed for the Janus program. Replicate exposures were begun, once a week, in late December, 1964, and were continued for 7 months, through July, 1965. Each replicate, consisting of about 50 males and 50 females, was run weekly. Some of the variables that might

affect the radiation response included: a) the sex, b) the age at irradiation, c) the number of mice in a cage prior to exposure, and d) the irradiation container.

PROGRESS REPORT

The results of these experiments are summarized in Tables 22 and 23. It is evident from these data that the $\text{LD}_{50/30}$ for the CF No. 1/Anl female mouse was reproduced over the 7-month test period. With one exception (G17, Table 23), this value varied from 920 to 983 R with relatively small standard errors. There was no significant difference in the $\text{LD}_{50/30}$ of the first run (December 1964) of 937 ± 5 R, and the last runs (June 1965) of 929 ± 12 R. The mean survival time of all female 30-day decedents was 15.2 days with an insignificant mortality prior to day 10 after the exposure.

At first, the males, showed a different pattern: The

TABLE 22. ACUTE RADIATION RESPONSE OF MALE AND FEMALE CF NO. 1/ANL MICE TO Co^{60} GAMMA RAYS

Sex	Number 30-day decedents/number mice irradiated	Time of exposure	Number of replicates	Deaths before day 10, %	Mean survival time of 30-day decedents, days
Female	438/945	12/15/64-4/21/65	19	0.4	15.2
Male (15/cage at weaning)	186/312	12/29/64-2/2/65	6	18.7	11.9
Male (4 littermates per cage at weaning)	67/213	3/23/-4/21/65	5	1.5	16.0
Male (isolated at weaning, 1/cage)	91/186	12/15/64-3/9/65	4	0	14.2

Summary: Irradiation of CF No. 1/Anl mice, both sexes, with Co^{60} gamma rays. High-Level Gamma-Room; time of exposure, 20 minutes; mice irradiated singly in plastic cartons; dose range, 780-1050 R; 19 replicates at weekly intervals.

TABLE 23. REPLICABILITY OF LD_{50/30} VALUES FOR CF No. 1/ANL MICE IRRADIATED
IN THE HIGH-LEVEL GAMMA ROOM WITH Co⁶⁰
(22 replicates, December 1964–July 1965)

Replicate No.	Irradiation date	Age of mice at irradiation, days	Number exposed		Preirradiation housing (mice/cage)	LD _{50/30}	
			Female	Male		Female	Male
G3	12/29/64	58	55	52	4	937.0 ± 5.5	859.7 ± 20.9
G4, 5, 6	1/5–1/19/65	105–115	173	155	4	937.3 ± 8.7	836.2 ± 24.9*
G1, 2, 9, 13	12/15/64–3/9/65	58–93	198	204	1	932.3 ± 11.6	869.3 ± 9.5†
G7, 8	1/26/65–2/2/65	105–110	110	105	4	957.3 ± 10.7	862.6 ± 71.6
G12	3/2/65	94	59	55	8‡	943.8 ± 2.3	842.8 ± 0.6
G16	3/29/65	86	48	46	4	966.3 ± 1.7	932.1 ± 12.6
G17	3/30/65	86	51	51	8‡	870.1 ± 89.9	915.0 ± 16.0
G18	4/6/65	79	50	28	4	919.9 ± 1.7	863.5 ± 13.7
G19	4/21/65	72	48	52	4	983.3 ± 34.4	932.3 ± 9.9
G20, 21, 33	4/21/65–6/29/65	80–100	72	—	4	929.3 ± 12.5	—
G15, 23, 28, 29	3/23/65–6/17/65	58–121	—	110	4	—	1006.0 ± 50.7

* See Figure 28.

† See Figure 29.

‡ Irradiated in plastic vials (not in cartons).

FIG. 28.—Acute radiation mortality of CF No. 1/ANL mice exposed to Co⁶⁰ γ rays.

FIG. 29.—Radiation response of both sexes of CF No. 1/ANL mice to Co⁶⁰ γ rays when mice were isolated at weaning.

LD_{50/30} figures ranged from 836 to 871 R, almost 100 R below those of the female mouse. No similar large difference in the acute response had been reported previously for both sexes. An example of these differences is illustrated in Figure 28. A much earlier mortality was also evident, with almost a fifth of the male decedents dying before the 10th day. This reduced the mean survival time by at least 3 days to 11.9 days (see Table 22).

We looked for the causal factors of this sexual difference. Many of the males in this early period showed skin abrasions. Following this observation, we found that it is a routine practice to randomize mice at weaning, 15 animals to a large plastic cage. They are maintained as stock animals under these conditions

until they are distributed to the investigators for experimental use. When 15 male mice are placed together, especially when they are from different litters, much fighting occurs and the less aggressive animals are frequently injured. Skin lesions that result may lead to infections which may influence their radiation sensitivity. Therefore, we introduced a new system: All males, when weaned, were placed four littermates per cage. Aggression was eliminated, no further skin lesions occurred, and early male mortality ended (see Table 22). Both the mean survival times and the $LD_{50/30}$ values became similar for both sexes. This change is reflected by the figures in the bottom portion of Table 23, beginning with replicate G16.

Several replicates were run in which mice were isolated at weaning, one animal per cage (see Table 22 and Figure 29). This procedure, as expected, reduced early mortality in the males. After March, 1965, the mean survival time of all male decedents was 15.0 days, not significantly different from that of the female mouse.

CONCLUSIONS

1. Animal behavior (aggression) may influence radiation sensitivity.
2. When fighting is controlled, there is no significant difference in acute radiation response between the male and female CF No. 1 mouse exposed to gamma rays, as judged by $LD_{50/30}$ values or mean survival times.
3. There was no large differences in acute radiation response in this strain of mouse between the 2nd and 4th month of age.
4. Whether the females are caged 1, 4, or 8 per cage prior to irradiation, does not change the $LD_{50/30}$ values significantly.
5. The plastic cages, developed for the Janus program, appear to be satisfactory.

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GONAECTOMY AND ACUTE RADIATION MORTALITY IN CF NO. 1/ANL MICE

Howard H. Vogel, Jr., Nicholas Bink, and Richard R. Barhorst

PURPOSE AND METHODS

In testing the replicability of the $LD_{50/30}$ (Co^{60} gamma rays) under various environmental conditions, it was observed that the male CF No. 1/Anl mouse was more radiosensitive, showed an earlier death pattern than the female, and that the $LD_{50/30}$ of this male mouse appeared to be 100 R lower than the comparative figure for the female mouse. Studies were undertaken to determine the cause of this sex difference. One obvious attack on this problem was to castrate both sexes and compare the $LD_{50/30}$ values of gonadectomized with normal males and females.

PROGRESS REPORT

The results of such castration experiments are summarized in Figures 30 and 31: 70 normal and 72 ovariectomized females and 110 normal and 105 castrated males were used in these exposures (850-1000 R). The gonadectomy was performed 2 to 3 weeks prior to irradiation. All mice were exposed for a constant time, 20 minutes, in the High-Level Gamma Room, when the animals were approximately 3 months old.

CONCLUSIONS

1. There was no significant difference between the $LD_{50/30}$ values of normal male (1006.0 ± 50.7 R) and

FIG. 30.—Dose-survival data in normal and gonadectomized female CF No. 1/Anl mice.

FIG. 31.—Dose-survival data in normal and castrated male CF No. 1/Anl mice.

RADIATION REPAIR: USE OF THE SPLEEN-COLONY METHOD AFTER PAIRED DOSES OF FISSION NEUTRONS AND Co⁶⁰ GAMMA RAYS

Howard H. Vogel, Jr.

PURPOSES AND METHODS

The paired-dose technique has been used by many investigators to study radiation recovery. The criterion used has been acute mortality. The spleen-colony method of Till and McCulloch⁽¹⁾ offered a new technique for studying early radiation repair of the hematopoietic system in the mouse. It is necessary, in radiation biology, to find a good correlation between cellular damage and radiation mortality.

PROGRESS REPORT

When CF No. 1/Anl female mice are irradiated with 800 R gamma rays from Co⁶⁰ and sacrificed 10 days after exposure, an average of about 3 colonies is found in their spleens. However, when such a whole-body dose is fractionated into two equal parts (400 R per dose), marked repair is noted within a few hours after radiation, as judged by either the mean number of colonies counted in the spleens, or the wet spleen weights, 10 days after exposure.

castrated male mice (974.4 ± 41.5 R), nor between the slopes of the dose-mortality lines.

2. There was no significant difference between the LD_{50/30} values of normal female CF No. 1/Anl mice (929.3 ± 12.5 R) and ovariectomized mice (919.5 ± 3.5 R).

3. There was a significant difference (*t*-test, 95% level) between the slopes of the normal and ovariectomized mice.

4. In 1957 we reported an LD_{50/30} of 929 ± 5 R for young adult female CF No. 1 mice exposed for 90 minutes to Co⁶⁰ gamma rays. It is of interest that, in 1965, this figure for CF No. 1/Anl female mice, irradiated for 20 minutes in the high-level gamma room was 929.3 ± 12.5 R.

5. It is clear from these experiments that the males, in these replicates, are no more radiosensitive than the female CF No. 1/Anl mice to acute gamma irradiation.

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Figure 32 illustrates the mean number of spleen colonies counted 10 days after such paired exposures to 400 R gamma rays. The interval between the doses was varied from 1 to 16 hours. Each of the 10 points represents 16 mice. It is evident that there is significant repair of radiation damage, as judged by a doubling of the colony number, when the interval between exposures is only 2 hours. Maximum repair occurs after about 5 hours when the colony number is approximately 4 times that resulting from the single dose. This maximum repair time, around 5 hours, has been reported by Till and McCulloch⁽¹⁾ in two other strains of mice, C57Bl/Ha and C3 Hf/Ha.

We are continuing this study of radiation recovery, using the 5-hour interval between varying gamma-ray doses.

We have also completed a similar series of fractionated exposures of mice to fission neutrons. In marked contrast to the gamma-ray experiments, there is no evidence of any repair after the first neutron dose, as

FIG. 32.—Radiation repair after x-rays

judged by numbers of colonies. The single neutron exposure and the paired fractions produce the same number of splenic colonies after 10 days. We have tested the time interval between the neutron exposures from 1 to 24 hours. This confirms and extends the preliminary work reported by Schneider and Whitmore,⁽²⁾ who reported that damage to mammalian cells

from fast neutrons "is not recoverable with time, at least over a period of 120 minutes."

CONCLUSIONS

The spleen-colony technique in CF No. 1/Anl mice appears to be useful for studying hematopoietic repair following gamma rays, but not following fission neutron radiation.

It appears possible to correlate hematopoietic damage after x-rays and gamma rays with mouse lethality.⁽³⁾ Kallman⁽⁴⁾ has reported a minimum recovery, after x-irradiation with 150–450 R, in C57Bl male mice to be between 4 and 8 hours, and a maximum to be between 8 and 12 hours.

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HEMATOPOIETIC REPAIR AFTER SINGLE WHOLE-BODY IRRADIATION OF MICE WITH FISSION NEUTRONS AND Co⁶⁰ GAMMA RAYS

Howard H. Vogel, Jr.

PURPOSE AND METHODS

We have carried out comparative studies in mice exposed to single whole-body irradiation with either fission neutrons or gamma rays from Co⁶⁰. In this work we have used the Till-McCulloch spleen-colony method⁽¹⁾ to compare the spontaneous or endogenous colonies found in the spleens 10 days after exposure to varying doses of the two external radiations. No marrow cells were injected into these mice. The colonies counted in the spleens were formed from cells which either escaped the effects of irradiation or recovered from these effects within the 10-day period. There is evidence that colonies are clones, each derived from a single stem cell.⁽²⁾

PROGRESS REPORT

We have confirmed earlier work by McCulloch and Till⁽³⁾ which reported a linear relationship between

gamma-ray dose and average number of splenic colonies 10 days after irradiation, when these are plotted on semilog paper. For the mouse used in our experiments, CF No. 1/Anl female, this relationship appears to hold, at least between 675 and 900 R (Figure 33). When the dose falls below 600 R, the number of colonies becomes very large and counting errors occur.

We have found a similar relationship between the wet weights of the spleens 10 days after exposure and the radiation dose. A close correlation was observed between the average number of splenic colonies counted and the mean splenic weights of the irradiated mice.

One of the most interesting comparisons between gamma rays and fission neutrons involves the size of the single dose necessary to reduce the mean colony number to only one or two colonies. A dose of gamma rays less than the LD_{50/30} (930 R) will produce this effect (Figure 33). However, it is necessary to give ex-

FIG. 33.—Endogenous spleen colonies counted 10 days after single, whole-body gamma irradiation of mice. (Dotted line in upper portion of figure indicates 30-day mortality: note scale on right vertical axis of graph.)

tremely high doses of fission neutrons in order to eliminate splenic colonies after a single acute exposure (Figure 34). A neutron dose equivalent to an LD_{50/30} (323 rads) will still produce, on the average, approximately 15 colonies in the spleen 10 days after exposure. After exposure to 360 to over 400 rads (above the LD_{99/30}) there is a wide variation in the number of colonies in

individual spleens, but a mean of at least 7 or 8 colonies is noted. Only when the extremely high level of 450 rads of fission neutrons is reached, is the mean colony number reduced to 1 or 2 per spleen. At this high dose, it is almost impossible to keep the mice alive for the 10-day period, even using antibiotic therapy.

From these experimental results, it appears that some hematopoietic stem cells must escape damage by neutrons and produce colonies in the spleen. This may be significant in future consideration of neutron-radiation therapy.

CONCLUSIONS

When the average number of colonies counted in the spleen 10 days after irradiation is compared after Co⁶⁰ gamma irradiation and after fission neutron irradiation, the neutrons are approximately twice as effective. In fact, an RBE of 1.9 multiplied by the neutron dose in rads gives a good fit to the gamma-ray curve. This RBE figure is significantly smaller than the RBE for acute 30-day lethality in this mouse, which is approximately 2.7 (873 rads/323 rads). It is even smaller than the relative biological effectiveness of these two radiations (6.3) for the production of equal probability of death on the 4th and 5th days after exposure, the time of the intestinal syndrome.

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FIG. 34.—Endogenous splenic colonies counted 10 days after single, whole-body irradiation of mice with fission neutrons. Note linear plot: LD_{50/30} was 323 rads for CF 1/Anl female mouse at CP-5.

demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature* **197**, 452 (1963).

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INCIDENCE OF MAMMARY NEOPLASMS IN RATS FOLLOWING SINGLE EXPOSURES TO FISSION NEUTRONS

Howard H. Vogel, Jr. and Robert Zaldivar

PURPOSE AND METHODS

Three years ago we reported the incidence of mammary neoplasms in female Sprague-Dawley rats irradiated with fission neutrons or Co⁶⁰ gamma rays at different dose rates.⁽¹⁾ The animals were irradiated with four weekly doses of either 100 rads of fission neutrons or 200 rads of gamma rays each week. The incidence of neoplasms was significantly higher and the life-span was considerably shorter in the neutron-irradiated rats. The relative biological effectiveness (RBE) (n/γ) for these criteria was, therefore, greater than two.

The late L. H. Gray⁽²⁾ of England suggested that rat mammary tumor incidence might be an excellent test system in which to compare the effectiveness of whole-body doses of external radiations differing widely in linear energy transfer (LET). This system has been extensively studied in the female Sprague-Dawley rat, after both x- and gamma rays, at Brookhaven National Laboratory.⁽³⁾ It seemed of interest to run a similar study using fission neutrons.

During early May, 1965, 96 female Sprague-Dawley/Anl rats, 2-3 months of age and *specific pathogen free* were exposed to single, whole-body doses of fission neutrons (with 10-15% gamma-ray contamination) at the CP-5 research reactor. These irradiated animals were randomly divided into three groups as shown in Table 24. Thirty-six unirradiated rats served as controls. These neutron doses were chosen as sublethal in the acute period. The LD_{50/30} for this species is approximately 380 rads under the experimental conditions at CP-5. All irradiations were carried out in the gamma-neutron radiation chamber⁽⁴⁾ at CP-5. This reactor was operated at 4.5 MW power; the boral filter plate⁽⁵⁾ was kept 55% open. The dose rate was 7-8 rads/min-

TABLE 24. RADIATION SCHEDULE FOR SPRAGUE-DAWLEY RATS EXPOSED TO 50-250 RADS OF FISSION NEUTRONS

Number of rats	Irradiation time, minutes	Total dose, rads
31	6.5	50
31	19.5	150
34	32.5	250

ute. All rats were rotated in the neutron beam in a plastic, motor-driven irradiation cage.⁽⁵⁾

PROGRESS REPORT (Table 25)

TABLE 25. INCIDENCE OF MAMMARY TUMORS 140 DAYS AFTER NEUTRON IRRADIATION

Dose, rads	Number of rats	Number of tumors	Number dead
Controls	36	0	0
50	31	8	1
150	31	7	0
250	34	8	2

The first tumor appeared, in the highest dose group, after 34 days. The first death occurred after 120 days.

Bond *et al.*⁽³⁾ have shown that mammary tumor incidence appears to be linearly related to dose, for both x- and gamma rays, between 25 and 400 R. This does not seem to be the case at present with neutrons. It is also of interest that 50 rads of fission neutrons has induced the same percentage of tumors (26%) 130 days postirradiation as 400 R of gamma rays. All animals in this series will be autopsied; tumor pathology will be reported by Dr. Zaldivar.

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THE JANUS PROGRAM FOR INVESTIGATING THE TOXIC ACTIONS OF NEUTRONS AND GAMMA RAYS

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PURPOSE AND METHODS

The objectives of the Janus program and the methods to be employed were described in the Annual Report for 1964.⁽¹⁾ The situation at present has not advanced materially beyond that described previously because of continued unavailability of the Janus facility and an extended shutdown of the High-Level Gamma Room. These delays have not decreased scientific productivity (and in some senses may have increased it) since the staff of the Janus program have been able to pursue their individual research projects with little or no hindrance. Since these projects are reported adequately under separate headings, the present report will deal only with a restricted set of topics not discussed elsewhere.

As noted previously, the Janus program has as a major mission the analysis of the late effects of continued low-level fast-neutron exposure. Experimental findings in the recently completed Gamma Ray Toxicity Program served to reinforce earlier evidence that the response of mammals to low-level exposure is not simply additive, but has strong interactions with other aspects of the environment and with the physiological state of the exposed population.

Consideration of these findings affected the eventual design of the Janus experiments in several important ways. It was decided, for example, that we cannot employ the previously accepted procedure in lifetime gamma irradiation studies, in which the irradiated groups lived in the irradiation room and their controls lived outside. Instead, it will be necessary to house all treatment groups in close proximity to one another, and to move the exposure groups to the irradiation facilities for each exposure, and to move the control groups to and fro in the same way at the same time. Further, it became necessary to conclude that the exposed populations cannot be permitted to be separated from their controls for more than $\frac{1}{3}$ of the time, or for periods of more than 8 hours repeatedly. It should be noted, however, that these stringent rules apply only to small doses, by which we mean doses that shorten life by less than 50%. At higher levels the response of irradiated populations is predominantly to the radiation exposure and is comparatively little affected by minor differences in the physical environment.

The decision that all treatment groups must share a common environment required the development of a

system for boxing, transporting and irradiating experimental animals. The cardinal requirements were that 1) the amount of neutron activation be sufficiently low, 2) the procedure have an acceptably low risk of bacterial infection, and 3) the initial and maintenance costs be as low as possible. All three requirements pointed to the same solution, which was to box the animals individually in small plastic ice cream cartons, transport them in the cartons to the irradiation room and back, and discard the cartons after returning the animals to their cages. For protracted or repeated exposures, the cartons can be irradiated stationary, since the random movements of the animals will equalize the exposure on the two sides of the body. Single brief exposures must however be delivered to the cartons while they are rotated on their vertical axes at a rate of a few revolutions per minute. Design and testing of a prototype system is now in progress.

The strongly interactive nature of late low-level radiation effects influenced the design of the Janus experiments in another important respect. A controlled environment animal facility will be built in a 4-room unit within the animal quarters. This will allow us to control ambient temperature over a 20°C range in two rooms with reasonably constant humidity. The intent is to introduce a significant magnitude of environmental modification without, however, probing the limits of tolerability. The temperature differential will enable us to maintain subpopulations of mice that differ by a factor of 2 in metabolic rate.

The 2 variable temperature rooms and 2 additional rooms adjacent will be equipped with a highly adjustable lighting system. Room illumination will be controllable over a 20-fold range of illumination intensities by a program clock and servoactuated dimmer. It will, thereby, be possible to control the light cycle at will. If a stable diurnal cycle can be established and maintained when the ratio of light to dark illumination levels is 10 to 1, it will be possible to carry out normal animal maintenance during the dark phase of a reversed light cycle. If necessary, the low light level can be supplemented with red light to which the retinas of nocturnal rodents are not sensitive because they contain only rod receptors.

These provisions will make it feasible to study radiation sensitivity, cell renewal, etc., as a function of the phase of the diurnal cycle. They will also allow us to

do exposures in two work shifts and still hold constant the phase of the diurnal cycle in which exposure is given.

An important change in animal maintenance will be instituted in the Janus program. Instead of the standard practice of making food available to mice continuously, we will withhold food from all animals in the program for 8 hours of every day. Two considerations make this necessary. First, there will be no food provided in the exposure cartons, and some treatment groups will be exposed as long as six hours. Second, the most important organ system in our histological and physiological research programs is the gastrointestinal tract, and experiments are planned in which a critical comparison is made between the pathophysiological effects of radiations on the gut and the incidence of gut death. We, therefore, requested Dr. Flynn of our Animal Quarters to develop and procure a cage that permits us to withdraw food by a single hand movement without removing the cage lid. At the same time as the modification of the food bin was under way, Dr. Flynn also carried through, at our

request, the development of a cage top which interposes a fiberglass filter between the cage interior and the outside world. It is expected that a group of four mice with all necessary food can be kept isolated from the animal room for 30 days. It will be necessary to change water bottles four times or so in that period, but even so the isolation will be far greater than has been attained in animal colonies previously.

With this degree of isolation available it becomes feasible to carry out a detailed analysis of the kinetics of acute radiation death and the quantitative relationship of morbidity to cellular and biochemical injury.

CONCLUSION

As noted above, the corporate Janus program has not yet begun and the individual projects are reported separately by the investigators listed at the beginning of this report.

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Cellular Effects

EFFECT OF CONTINUOUS IRRADIATION ON THE CELL CYCLE OF THE DUODENAL CRYPTS OF THE MOUSE

Samuel W. Leshner and George A. Sacher

PURPOSE AND METHODS

Previous experiments have shown that a sequence of well-defined changes occurs which alters the cell cycle pattern when the animal is exposed to continuous or long protracted daily irradiation. Following a single day's exposure, cells are delayed or actually blocked in G_2 . This delay is to some extent both dose and dose-rate dependent.⁽¹⁾ In addition to the delay in G_2 the number of cells in mitosis is reduced, the S period is lengthened, the whole cycle becomes more variable, and it is difficult to make a reliable estimate of the duration of the cell cycle. If the irradiation continues, the movement of cells through $G_2 + M$ returns to normal, as shown by the slope of the first rising arm of the curve and the time at which the 100% plateau is reached. How soon this part of the curve returns to normal is not known since following 50 R/day the curve is normal by 12 days and even after 220 R/day the curve is back to normal by 7 days. However, the duration of the generation cycle is reduced temporarily satisfying the increased demand for new cells. It is

this compensatory behavior of the duodenal crypt cells that makes it possible for the animal to survive even in the face of long, continued irradiation.⁽²⁾ Previous experiments have established the existence of the early delay in G_2 and M and the compensatory speeding up of the cell cycle, but it is still not known how soon the compensatory reaction takes over or how long it continues. Experiments are in progress which should give answers to both questions.

PROGRESS REPORT

As to how long the [accelerated] cycle can continue, a series of BCF₁ mice was exposed to 12 R per day for 570 days starting at 100 days of age, injected with 10 μ Ci of tritiated thymidine (TdRH₃), sacrificed at 1-hour intervals ranging from 1/2 to 20 hours, and autoradiographs of fixed duodenal samples were prepared for study. After 570 days at 12 R/day, less than 15% of the starting mouse population remains alive. The resulting percent-labeled mitotic figure curves of the irradiated and 670-day-old, nonirradiated controls in

FIG. 35.—Comparison of cell cycle of 670-day-old nonirradiated mice with cycle of animals exposed to 12 R per day for 570 days. The cell cycle of the irradiated mice ($10\frac{1}{4}$ hrs) is approximately 30% shorter than that of the control mice (15 hrs). Since $G_2 + M + S$ is the same in both groups, the decrease in cell cycle is due entirely to a drastic reduction in G_1 .

Figure 35 show that the cell cycle of the irradiated animals is $4\frac{1}{2}$ hours shorter than that of the controls. $G_2 + M$ is the same in both groups; hence, any reduction in total cycle time is due to a reduction in G_1 and S . Apparently the rate of cell production remains high owing to the accelerated cell cycle in surviving mice, at least at this low daily dose. We do have evidence from a previous experiment at a higher dose rate, 50 rads per $23\frac{1}{2}$ -hour day, that the cycle does lengthen in the long-term survivors.

On the premise that irradiation sensitivity increases with age, it was reasoned that the probability of detecting small, subtle changes would be greater in older animals. Young (100 days), middle-aged (400 days) and old (825 days) BCF₁ mice are being studied after exposure to 12 R/day for 1, 3, and 10 days in an attempt to obtain answers to such questions as, "Is there a measurable mitotic lag or G_2 block after a one day exposure?" Preliminary data indicate that crypt cells in the 400- and 825-day-old animals are indeed slowed in G_2 but there is little change in the 100-day-old mice.

How long after the start of exposure does the cell cycle become shorter?

CONCLUSIONS

All previous experiments indicate that the rapidly dividing crypt cells of the mouse small intestine increase production of new cells primarily through a shortening of the cell cycle without increasing the size of the proliferative population. The decrease in cell-cycle time is due to a reduction of S and G_1 but is principally due to a reduction or elimination of the G_1 period.

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DAMAGE AND RECOVERY IN DUODENAL CRYPT CELLS OF IRRADIATED MICE

Samuel W. Leshner

PURPOSE AND METHODS

The object of this series of experiments is to obtain 1) information concerning the extent and type of damage, 2) the relative radiation sensitivity of cells in the four stages of the cell cycle (G_1 , S , G_2 , M) at time of exposure, and 3) the rate at which this damage is repaired in the rapidly dividing crypt cells of the mouse duodenum following a single 300 R exposure to Co^{60} gamma rays. Previous experiments have established the duration of the cell cycle and the four phases, and have shown that, in the nonirradiated animal, this is an "all-out," steady-state proliferative cell population. Approximately 50% of the proliferative population of a nonirradiated mouse is always in S . This cohort of cells can be identified by labeling with tritiated thymidine (TdRH_3).

By injecting the TdRH_3 at a series of carefully selected times before and after irradiation, it is possible to study the effects of irradiation on a population of cells in a specific phase of the cell cycle at the time of exposure, e.g., 1) when TdRH_3 is injected immediately before irradiation, the labeled cells will represent those which were in S at time of exposure; 2) when TdRH_3 is injected 6 hours post-irradiation, the labeled cells will now represent a population which was for the most part, in G_1 at time of irradiation; 3) when TdRH_3 is injected at 12 hours postirradiation, most of the labeled cells would have divided once and be in S ⁽¹⁾ for the second time; at 24 hours they would have divided 2 times and be in S for the third time; at 48 hours they would have divided 4 times and be in S for the fifth time, etc.

A series of mice injected with 10 μc of TdRH_3 immediately before exposure and at 6, 12, 24, 48, and 96 hours postirradiation mice in each series will be sacrificed at close intervals ranging from 1/2 to 20 hours. Autoradiographs have been prepared and cell cycles were established by the percent-labeled mitotic figure method.⁽¹⁾ A study of the autoradiographs and the percent-labeled mitotic figure curves gives an estimate of the degree of damage, the relative sensitivity of cells in each of the phases at the time of irradiation, and the speed of recovery. Since it has been suggested that proliferative cells may be partially synchronized by irradiation,⁽²⁾ the number of cells in mitosis and in S (the number of labeled nuclei per crypt) will be determined.

PROGRESS REPORT

Immediately after 300 R whole-body Co^{60} gamma irradiation, the entry of new cells into mitosis (M) is blocked. Those in M complete the division process but the time spent in M is increased. It is thought that the cells continue to move through G_1 and S but that they are blocked when they reach G_2 . In a preliminary experiment, it was found that 75 R x-rays reduced the number of mitotic figures to a very low level but did not block cells completely in G_2 ; 150 R blocks cells in G_2 for 3 1/4 hours and 300 R blocks cells for approximately 4 1/2 hours⁽³⁾ (Figure 36). After 300 R Co^{60} gamma irradiation cells begin to move into M in small numbers 4 1/4 hours postirradiation (Figure 37, Graph B). Since it was found that over 60% of the first mitotic figures are labeled, it would appear that cells in S at time of irradiation are blocked in G_2 during the mitotic lag period and not at the end of S . When counts of labeled nuclei were made on crypt squashes following injection of TdRH_3 at close postirradiation intervals it was found that the number of labeled nuclei/crypt begins to drop immediately, indicating a slowing down of cells moving from G_1 into S (Figure 38). The experiment is far from complete; hence, it is not known when the number of labeled nuclei/crypt returns to near-normal values.

A careful examination of the percent-labeled mitotic figure curves shows that the so-called mitotic lag has been partially repaired and mitotic figures are now always present in most crypts; however, the movement of cells through G_2 is slow and does not return to normal values until 24 hours postirradiation. In addition, a 100% first plateau is not reached until 24 hours has past. This means that not only is G_2 longer in most cells but that a few unlabeled cells may go into M and not complete their division either because of extensive chromosome damage or because of some interference with the mitotic apparatus.

The duration of the cell cycle, as measured across the 50% levels in Figure 37, is 13 hours in the nonirradiated controls but is reduced to 10 1/4 and 11 1/4 hours respectively at 24 and 48 hours in irradiated animals. However, the cell cycle at 96 hours is longer than in the controls. All phases of the cell cycle are affected by irradiation, e.g., S , which is approximately 7 hours in nonirradiated controls, is reduced to less than 5 hours in the 6-, 12- and 24-hour postirradiation series but increases to 8 hours in the 4-day group.

FIG. 36.—Mitotic lag in duodenal crypt cells exposed to 75 R (○—○), 150 R (▲---▲), and 300 R (▽--▽) single-dose x-rays. Curves in the upper graph show that after 150 R and 300 R mitosis is completely blocked, while after 75 R a few cells are able to carry on division but at a much reduced rate. The lower graph shows that as soon as the block is repaired, cells that were in late *S* and *G*₂ at time of exposure go into division at the same time. This would support the hypothesis that the block occurs someplace in *G*₂ and not in *S*. Controls in lower graph represented by ●—●.

FIG. 37.—A, Cells of nonirradiated controls; B, mice exposed to 300 R Co^{60} whole-body, gamma irradiation (dose delivered in 15 min) and then injected with $10 \mu\text{C}$ of TdRH_3 (IP) at 10 minutes before irradiation; C, 6 hours postirradiation; D, 12 hours postirradiation; E, 24 hours postirradiation; F, 48 hours postirradiation; and G, 96 hours postirradiation. Cell cycles and the duration of each phase (S , G_2 , M , G_1) can be determined from the above percent-labeled mitotic figure curves for each series except for Graph B which will be completed as soon as the High-Level Gamma Room is again free. Although the complete mitotic block shown in Graph B ($4\frac{1}{4}$ hours) has been repaired by 6 hours, the movement of cells through G_2 and M is still retarded as shown by the slope and time of the first rising arm in Graphs C at 6 hours and D at 12 hours; $G_2 + M$ does not return to normal until sometime between 12 and 24 hours as shown in Graph E. The duration of S and the total cycle is shorter than control values at 24 and 48 hours but is longer at 96 hours; hence, at least one additional group is needed to attempt to find when the cell cycle returns to control values.

FIG. 37—Continued

FIG. 38.—Number of labeled nuclei per crypt squash. Curve (O—O) is a calculated curve which would be obtained if 300 R did not affect the movement of cells in *S* at time of exposure into *G*₂ and the movement of the cells in *G*₁ into *S*. Curve (●—●) shows the actual number of labeled nuclei at various times after irradiation, indicating a slowing down of cells moving into *S* from *G*₁.

CONCLUSIONS

All cells in all phases of the cell cycle are affected by ionizing irradiation although it would appear that cells in G_2 and M are the most sensitive and those in S the most resistant. Crypt cells in S at the time of irradiation are certainly heavily damaged, as shown by the number of aberrant mitotic figures, and the number of karyolytic bodies. Preliminary grain counts suggest that the rate of DNA synthesis in cells which were in G_1 at time of irradiation is reduced by a factor of about 4. Recovery, as measured by the ability of the cells to divide and synthesize DNA, is not as rapid in cells *in vivo* as that postulated by other investigators⁽⁴⁾ for cells *in vitro*; hence, it may not be possible to ex-

trapolate from mammalian cells *in culture* to proliferative cells *in the whole animal*.

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KINETICS OF CELL PROLIFERATION IN THE INTESTINAL EPITHELIUM IN NONIRRADIATED MICE

Samuel W. Lesher, Anthony R. Sallesse, and Marjorie A. Jones

PURPOSE AND METHODS

In order to evaluate the effects of irradiation, (acute or chronic) on any rapidly dividing cell population or cell renewal system, it is necessary to have accurate information concerning:

I. *Duration of the cycle and its phases, M , G_1 , S , and G_2 and a measure of the extent of their variability*

The accuracy of the percent-labeled mitotic figure method is undoubtedly the most reliable of *all available* methods. It can be improved by reducing the time interval between sacrifices and increasing the number of animals at critical parts of the curve, i.e., the first and second rising arms, the first descending arm, the first trough and the second plateau. In this series of experiments an effort is being made to obtain accurate information on 100- and 825-day old BCF₁ mice by reducing the sacrifice interval to 15 minutes and by increasing the number of animals at each interval to a minimum of four.

II. *The number of cells within the population which form the proliferative compartments*

For the small intestine, this means what portion of the total crypt? An estimate can be made if the duration of S and the total cell cycle and the number of cells in S are known. An estimate of the number of cells per crypt in S was made by determining the number of labeled nuclei 45 minutes after injection of

TdRH₃ in dissected-out, crypt-squash autoradiographs (ARGs).

III. *Classification of the type of population which is found within the proliferative compartment of the crypt*

Are these cells continuously-dividing, all-out cells or do they contain a G_0 component that acts as a reserve source of cells that feed into the actively-dividing complement to increase production when new cells are needed? To answer this question a multiple-injection technique was used. A series of 100-day-old BCF₁ mice was injected with 5 μ c of TdRH₃ every 2 hours. Forty-five minutes after each injection, two mice were sacrificed, and tissue samples of the duodenum were fixed and ARGs were prepared. When labeled nuclei/crypt squash are determined, the number of labeled nuclei should double within the proliferative compartment within a time interval equal to that of S . Because the duration of the S period is in excess of 50% of the duration of the total cell cycle, all of the nuclei within the proliferative compartment of the crypt should be doubled.

IV. *To what extent these factors vary in young old animals*

In a previous series of experiments⁽¹⁾ it was shown that the cell cycle of the CAF mouse increased with age. However, because our data indicate that cell cycles vary between strains, it was important to obtain cell cycle estimates on young and old BCF₁ mice. Cell

cycle estimates were made on BCF₁ mice at 55, 100, 300, 400, 670, 825, and 1050 days of age.

FIG. 39.—Cell cycles estimated by the percent labeled mitotic figures method for mice at 55, 100, 300, 670, and 1050 days of age. The cell cycle increases steadily with age ranging a little over 10 hours at 55 days of age to approximately 16 hours at 1050 days of age. The increase in time spent in *S* is equally well established going from 7 to 9 hours.

TABLE 26. CELL CYCLE OF MICE AT AGES 55–1050 DAYS

Age, days	Times in hours			
	Cell cycle	$G_2 + M$	<i>S</i>	G_1
55	10.2	1.5	7	1.7
100	13	2	6.7	4.3
300	14	2	8.0	4.0
670	15	2	8.5	4.5
1050	16	2	9	5.0

PROGRESS REPORT

Cell cycles have been completed for 55-, 100-, 300-, 670- and 1050-day-old mice and, as seen in Figure 39, the length of the cycle increases with age. Changes in the duration of the cell cycle are due to increases in the length of time the cells spend in *S* and G_1 , as $G_2 + M$ is approximately the same in all age groups (Table 26).

The data obtained by reducing the interval between sacrifices to 15 minutes and the number of mice per interval to a minimum of 4 are plotted in Figure 40. Three regression lines have been plotted for the first and second rising arms and the first descending arm. The low regression line was established by using only the low value for each time interval, the high regression line represents the high values and the middle line is based on the mean of all values. If estimates are made across the 50% levels the values for the cell cycle and its four phases shown in Table 27 are obtained.

The manner and extent to which these regression lines diverge and their decreasing slope is an indication of the degree of intercellular and interanimal variation in the time spent in the cell cycle and its phases.

Using the mean values as an acceptable measure of the duration of the cell cycle and its four phases, the size of the proliferative population within an intact crypt can be calculated if the following assumptions are made: 1) The proliferative population is asynchronous. 2) The cell cycle is essentially the same at all times of the day (no measurable diurnal effect). If the assumptions are accepted, then it can be further assumed that the proportion of cells in different stages of the mitotic cycle is the same at any time and is equal to the proportion of time of the whole cycle which that stage occupies.

Any of the four phases could be used in making this calculation but of the four phases only the number of cells in *M* and *S* can be determined. And our estimates of the duration of *M* are much more tenuous than our estimates of *S*. The number of labeled nuclei 45 minutes after injection of TdRH₃ is 66 with a SD of 8 (determined on crypt squashes).

FIG. 40.—Percent labeled mitotic figure determinations plotted against time for the BCF₁ mouse. Approximately four mice were sacrificed at 15-minute intervals at critical parts of the curve, namely, the first and second rising arms and the first descending arm. Three regression lines were run for each of these segments. The upper regression lines represent high values, lower regression lines represent low values and the middle line is based on all determinations.

TABLE 27. ESTIMATES OF CELL CYCLE BASED ON INTERVAL BETWEEN REGRESSION LINES (FIGURE 40) ACROSS 50% LEVEL

	Time in hours			
	Cell cycle	G ₂ + M	S	G ₁
Mean estimate	13 $\frac{1}{4}$	1.8	6.75	4.7
Minimum	12 $\frac{1}{4}$	1.75	6.3	4.2
Maximum	14 $\frac{1}{3}$	2	7.0	5.5

Substituting in formula

$$\frac{Sn}{PPn} = \frac{ST}{CCT}$$

$$PPn = \frac{66 \times 13.5}{6.75} = 130 \text{ cells/crypt in proliferative population,}$$

where Sn = number of cells in S , PPn = number of cells in proliferative population, ST = time spent in S , CCT = time of the total cell cycle.

The final question concerns the type of cells found in the proliferative population. Is it "all-out" or does it contain G_0 cells? Using the multiple-label technique, it was found that the number of labeled cells had more than doubled after the fourth injection, and no unlabeled nuclei were found within this zone (Figure 41). There is no evidence of a G_0 component within the proliferative compartment.

CONCLUSIONS

Experiments have shown that the duration of the cell

FIG. 41.—Number of labeled nuclei per crypt squash following 1, 2, 3, 4 and 5 injections. Solid line represents a calculated theoretical line based on a production of 10 cells per hour. The dotted line is a regression line based on actual counts.

cycle of the duodenal crypt cells in the BCF₁ mouse increases with age and that this increase is due primarily to changes in the duration of *S* and *G*₁. Reliable estimates for the duration of the cell cycle and for *M*, *G*₁, *S*, and *G*₂ have been obtained for the 100-day-old BCF₁ mouse. Our data show that without question the proliferative population is an "all-out" population

and does not contain a reserve of *G*₀ cells. If production is increased, the cell cycle must be shortened.

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CELL PROLIFERATION IN THE GALLBLADDER EPITHELIUM OF THE MOUSE

R. J. Michael Fry, Charlotte Weber, and Everett F. Staffeldt

PURPOSE AND METHODS

Sodium deoxycholate in the diet causes a marked increase in cell proliferation of the tissues in the gallbladder.⁽¹⁾ Tissues with a low cell turnover, but which can be stimulated to proliferate rapidly, are useful for some studies of irradiation damage. Such systems can also be used in the study of changes in cell metabolism that precede cell production. We have investigated some aspects of the proliferation of the epithelial cells of the gallbladder to see if they can be used as a test system.

Changes in proliferation have been assessed by determining, on autoradiographs, the number of labeled cells one hour after the injection of tritiated thymidine. We also wished to study the curve for the percent of mitoses labeled versus time after injection. To do this, BCF₁ mice (100 days old) were injected with tritiated thymidine. This group of mice had been given a diet containing 2% sodium deoxycholate which caused a greater degree of proliferation than the 1% diet. Two to five mice were sacrificed at hourly intervals between one and 11 hours, and at two-hourly intervals between 14 and 20 hours after the injection of the label. One hundred, or more, mitoses were examined for the presence of label (Figure 42) and the percent of mitoses labelled was plotted as a function of time.

PROGRESS REPORT

1. If 1% sodium deoxycholate is added to the diet, there is a latent period of more than 48 hours before the number of cells in the *S* period increases (Figure 43). The latent period is less than 24 hours, if dehydrocholic acid is given parenterally.

2. By spacing the periods of administration of the diet it is possible to produce a number of bursts of cell proliferation and to study the effects of repeated stimulation (Figure 43).

3. After the mice are returned to a normal diet, the proliferative activity falls to control level within three days.

4. Chromosome preparations can be obtained. The reason why the epithelium proliferates is not clear. It may be due to the toxic action of the choleretics directly on the cell. There is some correlation between the amount of distension of the gallbladder and the degree of proliferative activity. This suggests that stretching of the tissue may either contribute to or may be the cause of the changes that eventually lead to proliferation. This suggestion is supported by a marked increase in proliferation of the transitional epithelium of the bladder and ureter following ligation of the urethra.

The percent of mitoses labeled curve, as a function of time, is shown in Figure 44. It can be seen that the first part of the curve is similar to curves obtained for rapidly-dividing cell systems. However, no discrete second rising limb or peak is seen. The curve appears to "smear-out" after 8 hours; there is considerable inter-animal variation after 8 hours. As yet, we do not know how to interpret this curve, as there are a number of factors that remain to be investigated.

CONCLUSIONS

The gallbladder epithelium provides a test system, that may be useful for the investigation of 1) ploidy, chromosome aberrations, and proliferative capacity in relation to age and irradiation; 2) whether repeated periodic proliferation influences tumor incidence; 3) the relationship of proliferative activity to the induction of tumors by radiation. The use of choleretics has obvious advantages over ligation of the common bile duct,⁽²⁾ but we have found the variation in the response, particularly in relation to time, to be a troublesome feature. Either deoxycholic or lithocholic acid by intubation may prove more satisfactory.

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FIG. 42.—Photomicrograph of an autoradiograph. Autoradiograph of a squash preparation of gallbladder epithelium from mouse sacrificed 5 hours after the injection of the label showing two labeled metaphases.

FIG. 43.—The labeling index at various times after the onset of the special diet and when the period of diet was repeated. ○—○, Mice maintained on the diet for 2-6 days; ×—×, mice given the special diet for 6 days, returned to a normal diet for 8 days and then given the special diet for a second time.

FIG. 44.—Percent of mitoses labeled, as a function of time, for BCF₁ mice maintained on the diet for about three days. The average and the range of values for percent of mitoses labeled are shown.

RESPONSE OF NEOPLASTIC MAST CELLS (P815Y) TO X-IRRADIATION

G. Mark Kollmorgen and George A. Sacher

FIG. 45.—The percent labeled mitoses as a function of time for control, 62.5 R and 125 R. The times (hours) required to complete each of the four phases of the ascending limb are listed above each curve.

PURPOSE AND METHODS

A mouse neoplastic mast cell (P815Y) cultured *in vitro* has been used to study the effect of x-irradiation on the mitotic cycle. These cells grow in suspension and

increase exponentially with *all* cells dividing as indicated by separate measurements of cell cycle time and the doubling time. The elaboration of histamine and 5-hydroxytryptamine, as well as the uptake of specifi-

FIG. 46.—Cells collected with colcemide as metaphases after being labeled with tritiated thymidine

cally labeled precursors, indicates that the cells are also functionally differentiated.

Tritium-labeled thymidine is used to follow the movement of cells around the mitotic cycle (this report deals only with the ascending limb of the first cycle), and the percentage of labeled mitoses is plotted as a time function.

The curve has been divided into four time phases: 1) time required to observe the first labeled mitosis, 2) the time from the first labeled mitosis to constant slope, 3) the constant slope portion, and 4) the shoulder between constant slope and 100% labeled mitoses.

PROGRESS REPORT

Using doses of 125 R and 62.5 R which represent approximately an LD_{50} and $1/2$ an LD_{50} , the following alterations are noted: 1) the time required to observe the first labeled mitosis is increased, 2) the time from the first labeled mitosis to constant slope is increased, 3) the constant slope portion is decreased in extent (% labeled mitoses) but increased in time duration and 4) the duration of the shoulder between the constant slope portion and 100% labeled mitosis is increased (Figure 45). These data indicate that x-rays must have an effect on one or more of the following cohorts of cells: M , G_2 , or S .⁽¹⁾ Because of these results, a study of the disturbance to these cohorts of the population was undertaken.

When a dose of 125 R is given and the mitotic index is plotted, two observations are noteworthy; 1) a disturbance to the flow of cells into mitosis must occur near the end of the G_2 period, since the mitotic index falls immediately; and 2) the flow of cells into mitosis must be completely interrupted for 0.5 hour, since the extrapolated curve crosses the time scale at about 0.79 hour (the time normally required to complete mitosis). An increase in the mitotic time does not seem likely because the mitotic index drops at this same rate when higher doses of irradiation are used. These data indicate that M is not measurably lengthened with doses of radiation up to 125 R.

In order to follow the flow of labeled (S) and unlabeled (G_2) cells into mitosis, the mitotic figures were collected with colcemide⁽²⁾ (Figure 46). The collection with colcemide (0.321 $\mu\text{g}/\text{cc}$ of media) is temporarily complete as indicated by the following measurements:

1. A plot of $\log(1 + M/T)$ as a time function is a straight line for about 7.5 hours, with the time difference between $\log 1$ and $\log 2$ equal to the doubling time of about 12.5 hours. The population must be completely asynchronous and exponentially distributed around the mitotic cycle for this to be true.
2. The total population continues to increase for ap-

FIG. 47.—Collected mitoses per 1000 cells as a function of time after the addition of colcemide for control, 62.5 R and 125 R.

proximately the M period and then holds steady for 7.5 hours.

3. After an M period there are no mitoses advanced beyond metaphase.

The collected-mitoses curve for a dose of 62.5 R indicates a partial G_2 block for about 1.0 hour (Figure 47). The large difference in the slopes of the labeled (S) and unlabeled (G_2) cohorts indicates that cells in S require more time to reach mitosis. Whether the delay occurs in S or in G_2 is not known from these data.

A dose of 125 R shows very similar features. However, the flow of cells from G_2 to M is completely interrupted for about 0.5 hour followed by partial interruption for about 2.0 hours. The damage generally appears to be more extensive, and the time required for recovery is longer. The difference in rates of movement for the G_2 and S cohorts is again evident. Double labeling will help to clarify where S cells are delayed following x-irradiation.

CONCLUSIONS

The use of a metabolic inhibitor, colcemide, to collect these cells in metaphase is justified because the collection is complete. This collection process makes possible the study of flow rates of cells from labeled (S) and unlabeled (G_2) cohorts of the population.

The movement of cells from both of these groups into mitosis is disturbed in a dose-dependent manner.

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THE EFFECTS OF IONIZING RADIATION ON TENSILE STRENGTH OF RODENT VIBRISSAE

Melvin L. Griem* and R. J. Michael Fry

PURPOSE AND METHODS

The reasons for carrying out these experiments were as follows:

1. We require a relatively simple rapid test for investigating the effects of protracted low-dose neutron and gamma irradiation for studying the influence of irradiation on aging changes.

2. A localized narrowing of the hair shaft occurs following acute local irradiation. This narrowing can only be detected at doses of about 500 rads. We wished to investigate whether the measurements of tensile strength and elongation patterns would provide a more sensitive indicator. Furthermore if it proved a reasonable method, it might be used for RBE measurements.

While vibrissae may not provide the ideal system for testing tensile strength as there is some taper to the whiskers, they were chosen because they provided a nondestructive test and because of the characteristic and similar growth pattern of matched pairs of vibrissae as shown by Wright.⁽¹⁾ Thus, in acute irradiation studies one side of the snout can be irradiated and the corresponding vibrissae of the other side can be used as controls. Parallel studies on cell proliferation can be carried out and correlated with the physical and chemical characteristics of the hair. The changes in the whisker or hair following irradiation are the result of injury to the cells of the matrix which produce the medulla and cortex.

PROGRESS REPORT

To study the effects of x-irradiation the left side of the snouts of young adult Sprague-Dawley male rats were exposed to doses ranging from 50 to 1500 rads. The focal skin distance was 11.5 cm, 2 mm added aluminum filter was used, and the machine operated at 50 kV. This produced a beam quality with a half-value depth of 1.2 cm to tissue. The dose rate was 387 R per minute. The treatment cone contained a monitor chamber. Fourteen to sixteen days following irradiation

the vibrissae were identified and harvested by cutting them at the skin surface. The vibrissae were mounted on 1" x 3" cards with a 1/4" hole.

The stress-strain properties of the samples were investigated using an Instron materials-testing instrument, which was made available to us through the kindness of Mr. E. Ayers of the Instron Corporation. A load cell giving full deflection on the X-Y recorder either with 0-200 g or 0-500 g was used. The cross-head speed or elongation rate was set at 0.2 inch per minute.

An example of the recordings is shown in Figure 48. Elongation starts at point B, the initial yield occurs at point C, and breaking at D. The tensile strength is obtained from the deflection AD, and the "work to break"

FIG. 48.—Load-extension curves for paired rat vibrissae after 750 rads x-irradiation to the left side of the snout.

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FIG. 49.—Work to break as percent of control and percent of control tensile strength plotted as a function of dose

can be calculated from the area under the load-extension curve obtained from the integrator. Figure 48 also shows the difference in the recordings for paired control and irradiated (750 rads) vibrissae. The dose response curves for tensile strength and "work to break" are shown in Figure 49. It should be noted that there was considerable difference between paired vibrissae and also between animals both in the control and the irradiated groups. However, significant reductions in the strength of the vibrissae were found at 500 rads and above.

CONCLUSIONS

With refinements in technique, particularly by re-

ducing the length of vibrissae tested, it should be possible to increase the sensitivity of the method so as to make it useful for RBE determinations.

We are hopeful that the study of the physical characteristics of various tissues will provide useful quantitative data of both irradiation and aging changes. In the case of the lens previous studies on the effect of aging and irradiation have used opacification as the criterion of change. We hope that changes in the deformation characteristics will provide a more sensitive method.

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THE USE OF MULTIPLE BIOPSIES FOR THE STUDY OF THE CELL CYCLE OF THE MOUSE HAIR FOLLICLE

Melvin L. Griem* and R. J. Michael Fry

PURPOSE AND METHODS

In the study of the cell proliferation of normal tissue in rodents, individual animals have to be used for each experimental time interval, and, thus, the problem of interanimal variation is always present. This presents a particular problem in the study of the cell cycle by the fraction of mitoses labeled versus time method.

Adult Carworth Farms #1, female mice of the

Argonne strain, approximately 90 days old, were plucked over the back to induce the anagen phase of the hair cycle. Experiments were done at five days and ten days following this plucking. Intraperitoneal injections of 20 microcuries of tritiated thymidine were made at 10 P.M. in all animals. Because of the size of the mouse, largest number of biopsies done on one mouse was 11. (In the rat it is possible to obtain 30 or more biopsies of the skin. Additional experiments on rats are in progress). Ten days after plucking, one

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FIG. 50.—Percentage of mitoses labeled as a function of time interval between injection of label and biopsy. —●—, Data taken from a mouse five days after plucking; ○—○, data taken from several mice ten days after plucking. The following symbols were used for individual mice: ○, ●, ○, ●.

mouse was biopsied at 1, 1½, 2, and 2½, hours and hourly intervals between 3 and 7 hours after injection. Two mice were biopsied at two hours and at hourly intervals between 7 and 16 hours. Two mice were biopsied hourly between 16 and 24 hours. One animal was biopsied from 10 to 16 hours after injection of tritiated thymidine and 5 days after plucking.

The 4-mm punch biopsies were dipped in mounting fluid, frozen with a CO₂ quick freeze unit, and sectioned on a cold microtome at -30° C. Four-micron sections were made, fixed in 4:1 methyl alcohol-acetic acid fixative and then taken through graded solutions of ethyl alcohol to water. The slides were dipped in Kodak NTB 3 liquid emulsion, dried, and stored for one or two weeks in light-tight boxes, developed in D-19 developer for 1.5 minutes at 20°C, washed, fixed, and then stained with Mayer's haematoxylin and eosin. Labeled mitoses were counted in the region of the matrix in sections cut perpendicular to the skin, and longitudinal to the hair shaft. Proliferative cells in the dermal papilla and external root sheath were not counted.

PROGRESS REPORT

Figure 50 is a composite curve constructed from the data obtained from the several animals biopsied at various time intervals after injection of tritiated thymidine. The presence or absence of label was scored on between 100 and 300 metaphases observed in the follicles available in each sample. In some follicles it was noted that there were clusters of cells which appeared to be in the same phase of the cell cycle. The composite curve so obtained showed that the percentage of mitotic figures labeled reached 100% at 3 hours and remained at this level for 3 hours. Following this there was a rapid decrease in the percentage of mitoses labeled to a minimum between 10 and 11 hours. Subsequently, the

percentage of mitoses labeled curve rose to a second maximum of 96% at 18 hours. A third minimum point of 20% is reached at 22 hours. From this curve, an estimate of the *S* phase may be made from the time elapsed between the 50% points (at 2 hours and at 8 hours) resulting in an *S* period of 6 hours. A similar estimate of *S* is obtained between the 50% points on the second cycle (at 14 and 20 hours). If one takes the time elapsed between the 50% point on the first ascending limb to the 50% point on the second ascending limb, one obtains a cell cycle of about 12.5 hours. If the time elapsed from the origin of the first ascending limb to the beginning of the second ascending limb is taken, one obtains a cell cycle of about 9.5 hours. If the duration from the second minimum at 10.5 hours to the third minimum at 22 hours is measured, this yields a cell cycle of 11.5 hours.

CONCLUSIONS

It is of interest that there is a gross correlation between the data from various animals so that similar results were obtained at the same time interval irrespective of the number of biopsies that had been done previously. The percentage mitoses labeled at intervals between 10 and 16 hours was similar for the anagen hair follicle at both 5 and 10 days after plucking. This suggests that there is little or no change in the cell cycle during this period of anagen.

The data obtained at five days and ten days differ from previously published data in that there is a plateau on the first curve and there is less clear-cut dampening of the curve in the first 24 hours than previously reported by Cattaneo.⁽¹⁾

From these results, it would appear that there is little interanimal variation; however, it remains to be seen whether this will be so under other conditions. This method is being extended to study the whole

period from 0 to 30 hours in the hair follicle of rats. This technique may have application to comparison studies of the percentage of mitoses labeled curve under various conditions such as radiation, changes in temperature, and the like.

Physiological Effects

THE TISSUE MAST CELL

Douglas E. Smith and Sally V. Hass

PURPOSE AND METHODS

The mast cell, characterized by Ehrlich in 1879 as a unique element of the connective tissue, is packed with large cytoplasmic granules which stain metachromatically with some basic dyes. It contains and is a major source of heparin and histamine and in some species of 5-hydroxytryptamine. These compounds are of potential physiological significance in the normal animal and may cause vascular flow, clotting, and permeability defects in the irradiated animal. Enzymes concerned with the sulfation of heparin and decarboxylation of histidine, 5-hydroxytryptophane, and dihydroxyphenylalanine are also found in the cell. Work in this laboratory has centered on the morphology, origin, control, and function of this cell in the normal and irradiated animal.⁽¹⁾ During the past year, we have extended our electron microscopical study of the development of the mast cell in the foetal rat to include the regeneration of the mast cell and its cytoplasmic granules in the adult after treatment with histamine liberators. Following such treatment (e.g., with Compound 48/80, a polymer of *n*-methylhomoanisylamine and formaldehyde), the cytoplasmic granules may be released from the cell, a phenomenon which has been considered as a mechanism for secretion of the physiologically active products of the cell. Compound 48/80, 0.02% in distilled water, was given intraperitoneally in dosages of 150 and 200 μ g per 100 g of body weight

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six hours apart on day one. Controls were given similar volumes of distilled water alone. At two days and five days later, the rats were killed and tissues (skin of the paw and nose) were taken for electron microscopy. These were fixed in osmium tetroxide, embedded in Epon 812 resin, sectioned with the Huxley microtome, and stained in sequence with uranyl acetate and lead citrate prior to examination in the Siemens Elmiskop I electron microscope.

PROGRESS REPORT

Treatment with Compound 48/80 elicited a marked release of cytoplasmic granules from the mast cells of the skin of paw and nose. Electron microscopy revealed not only that individual granules but also that groups of granules may be set free in the surrounding tissue. In the latter case several granules appear within a set of bounding membranes (probably endoplasmic reticulum) and appear at varying distances from the mast cell. No signs of division of mast cells were found in this study. Regeneration of mast cells was apparent at two days and marked by an extensive cytoplasm (Golgi apparatus, endoplasmic reticulum, and ribosomes) and newly forming cytoplasmic granules.

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THE EFFECTS OF RADIATION UPON SURVIVAL OF THE HIBERNATING GROUND SQUIRREL

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and Marilyn A. Williams

PURPOSE AND METHODS

Because of its markedly low body temperature and metabolism, the hibernating mammal is a useful tool for studying the development of radiation damage. Although the initial energy-transfer reactions are not likely to be influenced by the range of temperatures involved in hibernation, one can expect that the subsequent chemical reactions would be temperature dependent. Thus, the development of damage leading to mortality is markedly slowed following lethal dosages of radiation to hibernating animals as compared with homeotherms.⁽¹⁾ In fact, when 80 to 100% lethal dosages are delivered during hibernation and the animals are kept in the cold for 3-4 weeks, no mortality occurs during this period. If the irradiated hibernators are then taken to and kept in a room at 23°C, they rouse from hibernation and mortality ensues on about the same schedule as if the animals had been irradiated on the day of their entry into the warm room. The purpose of the present experiments is to determine the possibility for recovery from a fractionated lethal dosage of radiation given during hibernation of the irradiated ground squirrel (*Citellus tridecemlineatus*). Data are also gathered on the radiation response of the ground squirrel for use as a background against which these and other experiments may be evaluated. The methods employed have been described previously.⁽²⁾

PROGRESS REPORT

In an initial experiment, it appeared that more recovery (as indicated by survival) occurred after 3 doses of 400 R of x-rays, over a 2-week period, to animals hibernating at 5°C than after similar irradiation of ground squirrels kept at 23°C. A single dose of 1200 R to hibernating animals gave 85% mortality after 30 days at 23°C.⁽³⁾ During the past year, no such differences appeared in a more extensive study in which hibernating and nonhibernating ground squirrels

were exposed to fractionated dosages totaling 1800 R of Co⁶⁰ gamma rays. As a part of the latter study, groups of seven squirrels were exposed to single dosages of 1300, 1400 or 1500 R while hibernating at 5°C, kept in the cold room for three weeks, and then transferred to a room at 23°C where they remained thereafter. Similar groups were always kept at 23°C and exposed to 1000, 1200, 1400 or 1500 R. In the former groups, no deaths occurred during the stay at 5°C. In the ensuing 42 days at 23°C, however, one or two animals from each group died. In the groups kept at 23°C, one, six, seven and six animals died during the first 28 days after 1000, 1200, 1400 and 1500 R, respectively. In the following 36 days, the final animal of the 1200 R group died. An explanation of these unusual results is not apparent at present. In further experiments on animals always kept at 23°C, it was established that the LD₅₀ at 30 days after total body exposure to Co⁶⁰ gamma rays is about 1060 R for the ground squirrel.

It must be noted, however, that a number of the ground squirrels collected for the work of the past year showed a syndrome characterized by abnormal motor activity, debilitation, coma, and death. This appeared to be related to brain lesions caused by an ascarid larva migrans.⁽⁴⁾ The animals in the above studies did not show signs of being so afflicted.

The work is being continued to elucidate the unusual effects of hibernation on the radiosensitivity of ground squirrels.

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VISCERAL LARVA MIGRANS (NEMATODA) CAUSING A CENTRAL NERVOUS SYSTEM SYNDROME IN THE THIRTEEN-LINED GROUND SQUIRREL (*CITELLUS TRIDECIMLINEATUS*)

Thomas E. Fritz, Douglas E. Smith and Robert J. Flynn

PURPOSE AND METHODS

For more than ten years, the thirteen-lined ground squirrel, *Citellus tridecemlineatus* has been collected by one of us (D.E.S.) in the fall for use in experiments on hibernation. Usually, 100 to 300 animals have been captured on nearby golf courses and fields by flushing them from their burrows with water. Each year, animals have died during the first day or two following capture, but only rarely afterward. Death has appeared to be caused by exposure and secondary pneumonia from wetting and chilling during trapping and the subsequent dipping for ectoparasites, as well as by trauma produced by fighting during transport.

In the fall of 1964, about 300 ground squirrels were captured from areas where others had been collected in prior years. Within two weeks following the early deaths, several squirrels showed abnormal motor activity followed by debilitation and coma, a syndrome not previously observed. An attempt was made to determine the cause of this sickness. During the ensuing weeks and months, additional animals showed these signs. They were segregated from the remainder of the squirrels and observed for varying periods of time before being killed and subjected to a routine necropsy examination. More than two hundred squirrels were subjected to gamma irradiation. Three of these showed abnormal motor activity, and one of them was examined.

Similar examinations were carried out on 10 untreated and upon 25 lethally irradiated (Co^{60} gamma rays) squirrels which showed no abnormal motor activity. The brain of each animal was removed and fixed in formalin. Other tissues were collected when lesions were observed. Tissue sections were prepared by the paraffin method and stained with hematoxylin and eosin.

PROGRESS REPORT

During the period from September, 1964 to June, 1965 twenty-eight untreated animals showed unusual motor action which ranged from a high level of spontaneous (but coordinated) running to distinctly abnormal motor activity. The latter included torticollis, opisthotonus, running or paddling movements while lying on the side or back, circling, and abnormal postures while lying or standing, particularly dorsal flexure

of the neck. Fifteen of these animals showed reactions of the latter type and lost weight progressively or became emaciated. Squirrels which showed only a high level of spontaneous running did not lose weight.

At necropsy, no significant gross lesions were observed in any of the animals examined except for secondary degenerative changes of the parenchymatous organs associated with emaciation and inanition and hemorrhagic lesions in the irradiated squirrels. About half of all animals were infected with from 2 to 20 nematodes in the small intestine. These were identified as *Rictularia citelli*.*

On microscopic examination, inflammatory lesions were observed in the brains of the fifteen animals which had shown abnormal motor activity and weight loss. Structures, morphologically resembling metazoan parasites, were present in nine of these (Figures 51 and 52). The parasites were identified as ascarid larvae.† The inflammatory lesions varied widely in severity and distribution but in general were most commonly located in the brain stem and cerebellum.

The brain of the one irradiated animal exhibiting abnormal activity also showed encephalitis but no parasites. No significant lesions or parasites were found in the brains of 25 animals which died following gamma irradiation and which had never displayed abnormal motor activity. The same was true of ten untreated ground squirrels which had never shown abnormal motor activity.

In general, the reactions were typical of those reported for a nematode invasion of the central nervous system.^(1, 2) The changes included necrosis and malacia with a granulomatous inflammatory reaction including formation of multinucleated giant cells, glial proliferation and perivascular infiltration of mononuclear leukocytes. The parasites were usually associated with the cellular reaction and malacia but in several instances a demonstrable response was absent. In six of the fifteen animals in which significant lesions were present, no larvae were demonstrated.

* M. B. Chitwood, Senior Research Parasitologist, Beltsville Parasitological Laboratory, A.R.S., U.S.D.A., Beltsville, Maryland, identified the nematodes as *Rictularia citelli*.

† M. B. Chitwood, Senior Research Parasitologist, Beltsville Parasitological Laboratory, A.R.S., U.S.D.A., Beltsville, Maryland and N. D. Levine, Professor of Parasitology, College of Veterinary Medicine, University of Illinois, identified the nematodes in these sections as ascarid larvae.

FIG. 51.—Demyelination associated with ascarid larvae in the brain.

FIG. 52.—Ascarid larvae in the brain

CONCLUSION

The demonstration of ascarid larvae in the brains of a number of these ground squirrels in the absence of adult worms in the intestine of any of them suggests that they are not a transitory stage in the development of a parasite in its definitive host but that they are incidental or accidental parasites or that they are in a transport or paratenic intermediate host.

The extent of migration by larval nematodes, both in "normal" and "abnormal" hosts has been recognized for many years, but its significance has only been appreciated recently through studies on several animal species.⁽³⁻⁵⁾ Sprent^(6, 7) reported on migratory behavior of larvae of various ascarids in mice and called atten-

tion to the frequent invasion of the brain. In the ground squirrels considered in this report, lesions and larvae were demonstrated in the brain of significant numbers of animals but these do not explain the abnormal behavior in other animals which yielded neither. It should be emphasized that the histologic examinations were, at best, only a limited survey of the brain and that many larvae and lesions would be missed by these examinations or might be present in the spinal cord or meninges, which were not examined.

That ground squirrels in this study showed signs of central nervous system disease for some length of time or that the onset of signs was delayed for up to several months after they were captured and maintained in a laboratory facility is not inconsistent with the observations of Beaver,⁽³⁾ Sprent,^(7, 8) and Nichols.⁽⁹⁾ Each of them recovered living *Toxocara canis* larvae from the brain of experimentally infected mice, some as long as two years after exposure. The brain, where a large portion of larvae localize in rodents, is particularly interesting in this respect because it has been shown that encapsulation is slow and in mice free larvae may exist for at least two years.

The morphology of the larvae in these ground squirrels is consistent with those of ascarids as described by Nichols.⁽¹⁰⁾ Nichols, however, limited his description to species of nematodes unlikely to occur in an ecological environment like the location where our ground squirrels were captured. Tiner^(11, 12) studied fatalities in rodents caused by larval ascarids in the central nervous system and showed that some species are much more pathogenic than others. He demonstrated that both the skunk ascarid (*Ascaris columbiana*) and the raccoon ascarid were particularly pathogenic, with the latter being a common cause of rodent fatalities. It would be reasonable to assume that the larvae described in our animals were either from the skunk or the raccoon, since both animals occur in the area where the squirrels were collected.

The authors wish to thank Marilyn A. Williams and David V. Tolle for their skillful assistance in the conduct of this work.

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AUXIN FORMATION BY PREPARATIONS OF REGENERATING RAT LIVER

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PURPOSE AND METHODS

Enzymatic biosynthesis of auxin in the plant is a sensitive locus of radiation damage. This enzyme complex, localized in the cytoplasm, converts tryptophan to the growth hormone indoleacetic acid (IAA). It is possible that the radiosensitivity is associated with a lability of DNA synthesis, one preceding and causing the other, or both deriving from a common biochemical lesion.⁽¹⁾

The auxin-forming enzyme also occurs in animal organs, and we have studied its physiology in the rodent liver to a limited extent.⁽²⁾ We find that the enzyme exhibits a circadian rhythm of activity in the liver of the mouse,⁽³⁾ as do stages of nucleic acid metabolism in the normal,⁽⁴⁾ regenerating,⁽⁵⁾ and tumorous⁽⁶⁾ rodent liver. Since the regenerating rat liver provides a temporal phasing of protein, RNA and DNA synthesis, as well as mitotic activity (cf. Reference 7), it occurred to us that a time study of the tryptophan-IAA system in the liver after partial hepatectomy might provide a clue to sequential dependencies. The present report describes a preliminary effort along these lines.

Sprague-Dawley female rats, 7 to 8 weeks old, were anesthetized with ether. The middle and left lateral hepatic lobes were removed after ligation of their portal supply vessels. Control animals were treated identically except that there was no ligation or tissue excision. At various intervals following surgery, animals were killed by cervical fracture; the right lateral lobe was quickly excised and stored under liquid N₂ until assay. Details on the determination of enzyme activity as zero-order rates are described elsewhere,⁽²⁾

except that here the fraction of the incubation mixtures that contained the IAA was resolved by column chro-

matography with Dowex-3 rather than by paper chromatography. Eluates containing the IAA were bioassayed by the Avena section test. In some instances the absorbancy at 280 m μ of these fractions was determined spectrophotometrically against known amounts of IAA.⁽⁸⁾ Every analytical series included blanks treated identically except that tryptophan was replaced by glycine as a substrate. Nitrogen was determined for each enzyme preparation by H₂SO₄-H₂O₂ digestion and direct nesslerization of TCA precipitates. Each experimental unit consisted of six animals with at least duplicate determinations from each liver sample. Analysis of variance (distribution of *F*) was used to assess statistical significance.

TABLE 28. ACTIVITIES OF THE TRYPTOPHAN-INDOLEACETATE ENZYME SYSTEM IN CELL-FREE HOMOGENATES OF REGENERATING RAT LIVER

Hours after surgery	Observed activity μ g IAA/ml enzyme/hr	Nitrogen mg/ml enzyme	Specific activity μ g IAA/mg N/hr
4	(H)* 0.28 $\pm$ 0.036 (C) 0.26 $\pm$ 0.039	(H) 8.64 $\pm$ 0.35 (C) 7.03 $\pm$ 0.81	(H) 33 $\pm$ 5.6 (C) 41 $\pm$ 7.3
8	(H) 0.28 $\pm$ 0.083 (C) 0.16 $\pm$ 0.013	(H) 8.55 $\pm$ 0.97 (C) 5.98 $\pm$ 0.48	(H) 37 $\pm$ 10 (C) 29 $\pm$ 3.8
18	(H) 2.63 $\pm$ 0.42 (C) 1.65 $\pm$ 0.34	(H) 7.56 $\pm$ 0.41 (C) 6.89 $\pm$ 0.40	(H) 360 $\pm$ 63 (C) 230 $\pm$ 44
24	(H) 0.50 $\pm$ 0.053 (C) 0.48 $\pm$ 0.071	(H) 6.76 $\pm$ 0.32 (C) 7.28 $\pm$ 0.41	(H) 76 $\pm$ 8.8 (C) 65 $\pm$ 8.3
36	(H) 0.36 $\pm$ 0.030 (C) 0.36 $\pm$ 0.017	(H) 6.91 $\pm$ 0.26 (C) 6.86 $\pm$ 0.34	(H) 52 $\pm$ 3.3 (C) 53 $\pm$ 3.8
48	(H) 0.35 $\pm$ 0.069 (C) 0.38 $\pm$ 0.043	(H) 6.74 $\pm$ 0.37 (C) 7.26 $\pm$ 0.29	(H) 52 $\pm$ 8.6 (C) 53 $\pm$ 6.0

* Resident Student Associate.

* (H) Partial hepatectomy; (C) Surgical control.

FIG. 53.—Ratios of mean enzyme activities and mean nitrogen contents, partial hepatectomy (H) over surgical control (C).

PROGRESS REPORT

Table 28 lists the mean enzyme activity and nitrogen per unit weight of liver, and the mean specific activity, at various times after surgery. The ratios of the means for regenerating liver to means for surgical control are shown in Figure 53. In the regenerating organ there is a relative rise in both enzyme activity and protein that returns to control levels in 24 hours. The differences in enzyme activity and protein per unit weight between regenerating and control livers are significant at the 5% probability level. The difference in specific activities, whose variances are quotients, barely escapes significance at the 5% level. It may be

suggested that activation of the tryptophan-indoleacetate enzyme takes place in the period before 18 hours, the period of protein and RNA synthesis. Hence the activation of DNA synthesis appears to occur after the rise in titer of the auxin-forming enzyme, and is not a mandatory antecedent to that rise in titer. A more detailed time study of the enzyme in the first 24 hours after partial hepatectomy would be desirable.

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FATE OF 5-IODODEOXYURIDINE¹³¹ (IUdR) IN WHOLE ORGANS AND ITS INCORPORATION INTO THE DNA OF REGENERATING LIVERS OF RATS FOLLOWING X-IRRADIATION

Agnes N. Stroud and Austin M. Brues

PURPOSE AND METHODS

It has been reported recently⁽¹⁻³⁾ that 5-iododeoxyuridine (IUdR), an analog of thymidine, is readily incorporated into the DNA of synthesizing cells when they are labeled with radioiodine and that IUdR is as effective as a tracer as thymidine in following DNA synthesis. The iodinated analog is more easily assayed in whole tissues when they are labeled with I¹²⁵ or I¹³¹ because of their deep-penetrating gamma rays than is tritiated thymidine, whose beta rays penetrate only a few micra into tissue. For this reason, I¹³¹UdR was

used as a tracer to study the inhibition of DNA synthesis in rats after partial hepatectomy and after x-irradiation to compare with and amplify earlier experiments with thymidine. The uptake of the iodine by the liver, spleen, part of the small intestine, thymus and thyroid has also been investigated in these experiments.

Sprague-Dawley rats, weighing on an average of 240 g, were divided into two experimental groups. One group of 20 received no irradiation, while another group of 20 received 350 R whole-body irradiation, two at a time, from a 250 kvp x-ray machine at a dose rate

of 100 R/minute, 5 to 10 minutes prior to partial hepatectomy. Two rats from each experimental group were sacrificed at intervals during liver regeneration (12, 18, 24, 36, 48, 72 and 96 hours); 2 hours before sacrifice each rat was injected with $I^{131}UdR$ at 0.88 $\mu Ci/100$ g body weight. The liver, spleen, small intestine, thymus and thyroid were removed and either the whole organ or an aliquot of it was used for the measurement of radioactivity directly. An aliquot of the liver was stored (deep frozen) until the DNA was extracted by the Schneider⁽⁴⁾ method. The radioactivity in the DNA was read directly after extraction and the amount of DNA per liver was determined by the diphenylamine reaction.⁽⁵⁾

PROGRESS REPORT

This preliminary study has shown that $I^{131}UdR$ is an effective indicator of DNA synthesis in cells during liver regeneration giving results comparable to those with tritiated thymidine. Table 29 shows the uptake of $I^{131}UdR$ into the DNA of liver cells at the intervals used, after partial hepatectomy. After irradiation, the onset of DNA synthesis is delayed about 6 hours after liver regeneration begins in the nonirradiated group.⁽⁶⁾ The peak of DNA synthesis for the nonirradiated group occurs between 24 and 36 hours after partial hepatectomy. Because no samples were taken in between, the exact time of the peak in the irradiated samples cannot be determined. In recent experiments, where samples were taken every 2 hours, the results show a very sharp peak without the plateau seen and described in most reports. These experiments will be published later.

Total I^{131} concentrations in samples of whole organs showed no significant differences between animals on the basis of radiation insult or of time between radiation insult or hepatectomy and the injection of $I^{131}UdR$, with the exception of those noted below. Mean values of specific activities, expressed as radioactivity per gram of organ relative to the amounts of $I^{131}UdR$ injected per gram of whole animal, two hours prior to sampling, were as follows: spleen, 0.67; intestine, 1.06; thymus, 0.78; liver (unirradiated) 0.63; liver (irradiated) 0.56; whole blood, 1.38. Values for the spleen, the intestine, and the thymus in the 12-hour x-rayed group were about one-half of those for the other groups combined, and the liver values 12 hours after hepatectomy were decreased in both groups (control, 0.31; hepatectomy, 0.29). Deviations can possibly be explained on the basis of early general physiological effects of radiation and, in liver, by partial hepatectomy. The thyroid retained $1.9 \pm 0.8\%$ of the injected iodine, reflecting uptake of circulating iodide. The

TABLE 29. DISTRIBUTION OF $I^{131}UdR$ IN THE DNA OF REGENERATING LIVER CELLS AFTER PARTIAL HEPATECTOMY OF NONIRRADIATED AND IRRADIATED (350 R) RATS

Hours after partial hepatectomy	Counts/min/mg DNA	
	Nonirradiated	Irradiated
12	22.9	18.1
18	48.3	16.3
24	159.0	33.6
36	291.4	203.0
48	67.6	65.6
72	113.4	147.2
96	88.0	37.1

animals were not "loaded" with iodide before administering the tracer.

The DNA isolated from liver contained a very small proportion of the total I^{131} present in the organ; in no case did it exceed 6%. On the assumption that the actual liver content of DNA is 2.5 mg/g, which appears to be near a maximum value, the total DNA was calculated to contain no more than 11% of the total liver activity in the most active samples. In view of the variance in total organ content between animals, it may be concluded that at 2 hours after an injection of labeled IUdR, the total activity in the liver does not indicate the amount that is incorporated in DNA.

A series of long-term experiments using $I^{125}UdR$ carried out to study the persistence of the iodine in nucleic acid in liver and spleen will be reported in full at a later date. Some days appear to elapse after injection before the IUdR incorporated during rapid DNA synthesis is restricted to the DNA fraction.

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SOME ASPECTS OF AMYLOIDOSIS IN MICE

Marietta Miller, Everett Staffeldt, Eugenia M. Cooke and R. J. Michael Fry

PURPOSE AND METHODS

This report is divided into two parts: 1) the incidence of amyloid in the ileum of mice of various ages and strains, and 2) the problem of plasma determinations in mice with amyloidosis.

The literature on amyloidosis in mice is abundant and includes its relation to aging,⁽¹⁻³⁾ irradiation,^(4, 5) and strain.^(1, 3, 6) In our experience, amyloid deposits localized to the terminal portion of the ileum are common. There is, however, little information in the literature about amyloidosis in this site. Thung⁽²⁾ found the distal parts of the small intestine more frequently affected than the duodenum, whereas Dunn⁽³⁾ reported infiltration in the stomach, Brunner's glands and the colon, as well as in the intestinal villi.

The strains and ages of the mice that have been investigated are shown in Table 30. A further series, included in Part 2, was used for investigating the correlation between the presence of amyloid in the ileum and other sites. Identification of amyloid was made in each case by one or more of the following methods: 1) the thioflavine-T fluorescence method,⁽⁷⁾ 2) the periodic acid Schiff reaction, and 3) methyl violet. Thioflavine-T is not specific for amyloid, but with care it is a very useful method.

PROGRESS REPORT

Part 1

Age, sex and strain are all factors influencing the incidence of the lesion (Table 30). The higher incidence in the male LAF₁ mice is in agreement with observations made at this laboratory on the incidence of amyloid in other organs.⁽⁵⁾ The other findings of interest are: 1) in the cases studied, the amyloid deposits were not found proximal to about 8 cm from the ileocecal junction; 2) amyloid may be present in the ileum and not in other organs, and vice versa; and 3) although the incidence of amyloid in some organs is increased in mice exposed to 6 and 12 R/day gamma irradiation,

^(4, 5) no increase of the incidence of amyloid in the ileum has been found so far. Probably the reason for this is that the lesion in the ileum occurs considerably later in life than in other organs.

Part 2

Some years ago we found that LAF₁ mice with skin lesions had an apparent increase in plasma volume. The skin lesions were not, in most cases, thought to be of sufficient severity to explain the results. Furthermore, at necropsy evidence of amyloidosis was found and was confirmed in a number of the mice. We have determined plasma volumes in mice of various age groups with and without skin lesions and amyloidosis. Another stimulus to this investigation was the conflicting evidence on the use of the disappearance rate of either Evans blue (T 1824) or I¹³¹ albumin from the plasma as a diagnostic test for amyloidosis.^(8, 9) Plasma volumes were measured using the T 1824 and I¹³¹-albumin methods. In most of the tabulated results, we have chosen to compare the [(cpm/ml of plasma)/cpm of injected dose] × 100 in mice with and without amyloidosis or skin lesions. The comparison of the results of the plasma volume determinations by the two methods are shown in Table 31. The results by the T 1824 method were found to be significantly higher than by the I¹³¹ method. This finding is in agreement with those of Wish *et al.*⁽¹⁰⁾ The small sex difference in plasma volume can be accounted for by the differences in weight. The plasma volumes in the mice with amyloidosis are significantly higher than those without amyloidosis (Table 32). The values for [(cpm/ml of plasma)/cpm of injected dose] × 100 are higher in normal animals than in those with either skin lesions or amyloidosis. These results indicate that either the plasma volumes are increased, or that the leakage of the labeled albumin is greater in the mice with lesions. When amyloid was present only in the ileum, the results were within normal limits.

TABLE 30. INCIDENCE OF AMYLOID IN ILEUM

Strain	Age, days	No. of mice	No. of mice with amyloid	% Incidence
LAF ₁	688-749	27 (♂ 14, ♀ 13)	14 (♂ 11, ♀ 3)	52 (♂ 79, ♀ 23)
LAF ₁	800-900	18	17	94
BCF ₁	1000-1100	29	23	79
CAF ₁	900-970	54	8	15

TABLE 31. PLASMA VOLUME OF 250-DAY-OLD LAF₁ MICE

Sex	Method	No. of mice	Plasma volume, cc	Standard deviation
1. ♀	T 1824	13	1.49±	0.14
2. ♀	I ¹³¹ albumin	13	1.24±	0.23
3. ♂	I ¹³¹ albumin	10	1.45±	0.26

Group 1 differs from group 2 at the 0.01 level of Student's *t* statistics.

TABLE 32. THE RELATIONSHIP OF PLASMA VOLUME TO THE PRESENCE OF AMYLOID IN MICE

Condition	No. of mice	Plasma volume, cc	(Cpm/ml of plasma)/Cpm of Injected dose $\times 100^*$
1. Amyloid present in one or more organs	9	2.38 ± 0.52	
2. No amyloid found	6	1.38 ± 0.12	
3. Amyloid in ileum and not elsewhere	5		59.9 ± 15.1
4. Amyloid present but not in ileum	15		42.4 ± 6.7
5. Skin lesions	21		43.9 ± 6.2
6. No skin lesions or amyloid	17		61.5 ± 12.1
7. Amyloid present in one or more organs	20		46.5 ± 11.3
8. No amyloid found	11		63.9 ± 11.8

* Values below 50.0 are abnormal.

Groups 7 and 8 include mice of Groups 1 and 2, respectively.

Groups 1 and 2, 3 and 4, 5 and 6, 7 and 8 differ at the 0.01 level of Student's *t* statistics.

CONCLUSIONS

The study of senile amyloidosis of the ileum may be useful in testing certain facets of the immunological theory of aging.⁽²⁾ Neither the pathogenesis nor the reason for the strain differences is clear. From our re-

sults, we believe that there is increased passage of albumin from the blood compartment in mice with amyloidosis.

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ACUTE SIMPLE OBSTRUCTION IN THE RAT

Burt Kraft,* Harold Sutton, Patricia C. Brennan, and R. J. Michael Fry

PURPOSE AND METHODS

In this report, we give the results of the initial investigations into the effect of diverting the bile and of antibiotic treatment on the survival time following acute simple obstruction in the rat. These experiments are part of an investigation into the roles of bile, bacteria and motility in the cell turnover of the intestinal mucosa. Jenkins⁽¹⁾ found that diversion of the biliary, pancreatic and duodenal secretions to a point below the site of the obstruction increased survival time in dogs from 2-7 days to 12-33 days. Barnett and Clippingier⁽²⁾ increased the survival time in dogs with closed loop strangulation obstruction from 29 hours to 56 hours by the administration of penicillin. Amundsen and Gustafsson⁽³⁾ found the mean survival time was 240 hours in germfree rats with a low ileal strangulation obstruction compared to 44 hours in conventional animals. The role of bile in the pathological changes

following acute obstruction is not clear. Possible answers to this question include 1) the loss of fluid and electrolytes in the bile 2) the effect of bile on the permeability of the mucosal cells and 3) the effects of the toxic bile acid metabolites resulting from microbial action.

The various experimental procedures or treatments are shown (Table 33) with the number of animals used in the seven groups. Young adult ANL male rats were used. The various operations were carried out by standard procedures. The length of obstructed gut was standardized by closing the lumen with a suture at the level of what corresponds to the ligament of Treitz in man. In the Neomycin-treated groups the antibiotic was given in the drinking water, (2 mg/ml), for three days prior to operation and postoperatively.

PROGRESS REPORT

The results are shown in Table 33. It is clear from these results that either diversion of bile to a point

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TABLE 33

Treatment	No. of rats	Mean survival time, hrs	Standard deviation
1. High obstruction	7	28.5	± 5.7
2. High obstruction and Neomycin	4	26.2	± 1.2
3. High obstruction and common bile duct tied	4	73.3	± 10.2
4. High obstruction and common bile duct tied plus Neomycin	6	102.5	± 30.9
5.* High obstruction and bile bypass	7	54.2†	± 29.1
6.† High obstruction and bile bypass	3	64.4	± 22.7
7. High obstruction and bile bypass plus Neomycin	4	65.9	± 13.3

Group 1 differed significantly from groups 3, 4, 6 and 7 at the 0.01 level of Student's *t* statistics. ($p < 0.001$, $p < 0.001$, $0.01 < p < 0.001$, and $p < 0.001$ respectively.)

* Group 5, high obstruction and bile bypass carried out at same operation.

† Group 6, high obstruction was carried out three weeks following the initial operation to establish the bile bypass.

‡ One of the rats included in this group died at 17 hours after operation. The cause of death was not obvious but it probably was not the obstruction.

distal to the obstruction or preventing bile from reaching the intestinal lumen increases the survival time. Further experiments with different antibiotics would be necessary before a definite statement about the importance of bacteria could be made. However, these

results do not indicate that bacteria are implicated in the cause of the early deaths. The amount of I^{131} albumin in the fluid of the gut obstructed for 15 hours was determined 2 hours after the injection of the labeled albumin in animals from both the simple obstruction and the simple obstruction plus common bile duct tied groups. No significant difference was found. These results suggest that the presence of bile within this period (15 hr) does not increase the permeability of the mucosa for albumin.

CONCLUSIONS

On the evidence obtained so far, it appears that the bile contributes to the loss of fluid and electrolytes. The short length of obstructed gut is unable to absorb the combined volume of secretions and water which is drunk by the rats postoperatively. The excess of unabsorbed fluid causes marked distension. Neither the histological appearance nor the examination of the flora of the obstructed loop has shed any light on why diversion of the bile increases survival time. The obstructed segment of gut does provide a test system for the investigation of the interaction of bile and bacteria.

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MODIFICATION OF ANTIBODY PRODUCTION

William H. Taliaferro, Lucy G. Taliaferro, and Charles W. Kim*

PURPOSE AND METHODS

As outlined in the report of last year, the nature of immune mechanisms is being analyzed. Three systems have been used, i.e., the Forssman hemolysin response in rabbits, the antibodies arising in rats in response to *Trypanosoma lewisi*, and delayed hypersensitivity to trichina antigens in the guinea pig. Salient references may be found in the report of last year.

* Dr. C. W. Kim worked on a special N.I.H. Fellowship at Argonne and at the University of Chicago. His current address is Brookhaven National Laboratory, Upton, Long Island, New York.

PROGRESS REPORT

A. Effects of X-rays on Hemolysin Production William H. Taliaferro and Lucy G. Taliaferro

Radiation-induced effects on the initial gamma-1 hemolysin response in rabbits were reported last year. The secondary gamma-1 Forssman hemolysin response is now being intensively studied with respect to x-ray effects using doses extending from 25 R to 700 R. The response is being initiated by reinjecting a standardized number of sheep red blood cells before and after each test dose of irradiation.

B. *The Effect of Nucleic Acid Analogs on the Hemolysin Response*

William H. Taliaferro and Lucy G. Taliaferro

It has been reported that 5-bromodeoxyuridine (5 Br) and other base analogs may be incorporated into DNA of various cells in place of the natural component thymidine and that such cells become highly susceptible to various types of irradiation.

We started with the assumption that this analog might behave as a radiomimetic substance, i.e., would enhance or depress antibody formation depending upon the time of administration relative to antigen injection, or that it might restore to a varying degree antibody-forming capacity lost as a result of previous x-irradiation. For example, Jaroslow and Taliaferro have found that colchicine possesses 1) a marked adjuvant activity when injected with the antigen into unirradiated rabbits and 2) a marked restorative activity when injected with the antigen into rabbits irradiated a day or two previously with 400 R.

A study of the effect of 5 Br on hemolysin formation in rabbits with and without irradiation has now been completed. As compared to controls, the drug produces an adjuvant effect when given with the antigen to unirradiated rabbits, but it did not alter hemolysin-forming capacity as a result of previous or subsequent irradiation.

A brief account of the work follows. Large multiple doses of 5 Br (0.1 to 0.25 g/kg rabbit) were injected intraperitoneally into groups of rabbits at intervals from 45 minutes before to 2 hours after the intravenous injection of sheep red cells. The rabbits were either unirradiated or irradiated with 400 or 500 R the day before or the day after the injection of the drug and the antigen. Small doses of 0.01 g/kg rabbit within an hour before and after injecting the antigen were also tested in two of the categories. These experiments involved hemolysin titrations (50% end point) 3 times a week for a month or more in each of 60 experimental rabbits and 49 controls. The data were analyzed statistically with respect to 8 parameters of the hemolysin response. Using one of the most sensitive of these criteria, i.e., peak titer in log units, the following results were obtained.

Large and small amounts of the drug when injected with the antigen into unirradiated rabbits resulted in a significant increase in mean peak titer (3.84 ± 0.10 log units: $P = 0.009$ and 3.78 ± 0.08 log units: $P = 0.015$, respectively) as compared to the mean peak titer in unirradiated controls (3.48 ± 0.08 log units).

This enhanced effect of 5 Br on peak titer was not additive or restorative in respect to x-rays when in-

jected with the antigen either *before* or *after* total-body irradiation of the rabbits. Thus, large amounts of the drug, when injected with the antigen one day before the rabbits were given 400 R, resulted in the same increase in hemolysin as in irradiated nontreated rabbits (4.0 ± 0.10 vs. 3.8 ± 0.10), whereas large amounts of the drug when injected with the antigen one day after the rabbits were given 400 R resulted in the same depressing effect as in irradiated nontreated controls (1.75 ± 0.18 vs. 1.9 ± 0.13).

C. *Natural Hemolysin in Irradiated Rabbits*

William H. Taliaferro and Lucy G. Taliaferro

The preceding hemolysin experiments were elicited in rabbits by injecting foreign red cells that contain the widespread Forssman antigen, but rabbits develop small amounts of hemolysin probably as a result of absorbing minute amounts of the Forssman antigen in food, etc.

This so-called natural hemolysin in heavily-irradiated (500–600 R) rabbits was found to decline for about two weeks, then to increase temporarily in excess of normal amounts in one or two waves during the subsequent 2 or 3 months. A return to normal values occurred about 4 months after irradiation. Radiation with 300 R produced similar but smaller changes. This work has been published.⁽¹⁾

D. *Trypanosoma lewisi in the Rat*

William H. Taliaferro and Lucy G. Taliaferro

We are continuing work on ablastin, the antibody that inhibits nucleic acid and protein synthesis in rats, with especial reference to the possibility of restoring its formation after loss by x-irradiation.

E. *Delayed Hypersensitivity to Trichina Antigens in the Guinea Pig*

Charles W. Kim

Classical types of antibodies have been repeatedly shown to arise during infection of animals with *Trichinella spiralis* and have been shown to act adversely on the parasites when passively transferred with serum. There have, however, been no reports of the delayed type, i.e., the tuberculin type of hypersensitivity, that has been adequately controlled in respect to the presence of serum antibody.

The following true cellular hypersensitivity has been found that fulfills the following cardinal characteristics of cellular hypersensitivity. When guinea pigs were immunized with the trichina antigen with Freund's adjuvant, delayed hypersensitivity arose before serum antibodies could be demonstrated by the highly sensitive passive cutaneous anaphylaxis (PCA), and prep-

arations of the skin indicated a predominantly mononuclear response that is characteristic of delayed hypersensitivity. This work is being prepared for publication.

F. Antibody Synthesis in Irradiated Rats

M. M. Simić, V. S. Šljivić, M. Z. Petković, and D. M. Ćirković

This comprehensive monograph is in the process of being edited by W. H. Taliaferro and L. G. Taliaferro. It is to be published by the Boris Kidrich Institute of Belgrade, Yugoslavia. This venture is being carried on

because Dr. Simić worked at the University of Chicago while W. H. Taliaferro and L. G. Taliaferro were there and extended their hemolysin and irradiation work on the rabbit to the rat. The results are important intrinsically and comparatively, and it is hoped that this English version will facilitate their usefulness in the field of radiation biology.

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THE RESTORATION OF HEMOLYSIN PRODUCTION IN X-RAYED RABBITS

Bernard N. Jaroslow and William H. Taliaferro

PURPOSE AND METHODS

The induction of hemolysin-forming capacity lost in previously x-rayed rabbits can be restored to varying degrees by various substances. One of the most potent of these is colchicine. The effect of this drug has been intensively studied in groups of rabbits irradiated with 400 R (restorative action) and in groups not irradiated (adjuvant action) by varying the amount of x-rays (25 to 700 R), of antigen ($10^{5.2}$ to $10^{10.2}$ sheep red blood cells/kg rabbit), and of colchicine (0.067 to 1.8 mg/kg rabbit) and by varying the time of injecting the large dose of colchicine before and during the hemolysin response.

PROGRESS REPORT

Mean values $\pm$ standard errors for 5 parameters of the hemolysin response indicated the following as compared to control groups not given colchicine. 1) Col-

chicine in progressively increasing amounts when injected with antigen stimulated the production of more hemolysin in both irradiated (400 R the day before) and unirradiated groups, but the speed of the process was delayed as in the irradiated control group. 2) The largest dose of colchicine when injected with the antigen was decreasingly active as the x-ray dose was increased (50-700 R the day before), but was strikingly more active in the asymptotic region of the response as the antigen dose was increased in irradiated (400 R the day before) as well as in unirradiated rabbits. In addition, the drug was most active when injected during a 12-hour period ending with simultaneous injections of it and the antigen in unirradiated rabbits and during a 36-hour period ending 24 hours after the injection of antigen in irradiated rabbits. It was progressively less active in both groups for the following 3 days and essentially inactive at 6 days. This work is being prepared for publication.

ANTIGEN LOCALIZATION IN IRRADIATED RATS

*Bernard N. Jaroslow and Gustav J. V. Nossal**

PURPOSE AND METHODS

Studies of the effects of radiation on the immune response, using the parameter of antibody-forming capacity, have shown that one of the radiosensitive phases in the process of antibody formation is asso-

ciated with the induction of the immune response.⁽¹⁾ Since the pattern of antigen localization in lymph nodes has been shown to be related to antibody-forming capacity,⁽²⁾ we examined this parameter, which must surely be one of the earliest, if not the initial step of the induction of the immune response. To determine whether procedures that restore antibody-forming capacity also alter radiation-induced changes

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in antigen localization, we tested two known restorative procedures, i.e., 1) the injection of colchicine at the time of antigen injection, one day after x-rays; and 2) irradiation 2 days after antigen injection.⁽¹⁾

Our experiments were performed on Wistar albino rats injected with flagella or flagellin prepared from cultures of *Salmonella adelaide*. Antibody titers were determined by the bacteria immobilization test; and antigen localization was studied in autoradiographs of sections of spleen, lymph node, and thymus from rats injected with I^{125} -labeled antigen. The techniques of antigen preparation, autoradiograph preparation, and antibody titration were described previously in detail.⁽³⁻⁶⁾

PROGRESS REPORT

When a preparation of I^{125} -labeled flagella is injected into the footpads, much of it is sequestered in the popliteal lymph nodes, and lesser amounts are retained in the spleen and aortic lymph nodes. In the normal rat, the label is localized along the medullary cords and in the follicles of the lymph nodes (Figure

FIG. 54.—A, popliteal node of normal rat; B, popliteal node irradiated rat (450 R). Note the heavy labeling in the follicles (FOL) and medullary cords (MC) of both nodes and the cortical rim (CR) of localization in the irradiated node. Nodes were excised 2 days after antigen injection.

FIG. 55.—Popliteal node from a rat exposed to 8,000 R and excised 2 days after antigen injection. Note that the only remnant of follicular localization (FOL) is an occasional thickening of the cortical rim of localized antigen.

FIG. 56.—Spleen from a normal rat 3 hours after intravenous injection of antigen. Note the discrete rings ("target-distribution") of antigen in the marginal zone (MZ) and germinal center (GC) of the white pulp.

54A). When a secondary germinal center is present, it localizes as a crescentic cap around the germinal center. In rats exposed to 450 R one day before antigen injection, the label also localizes in the follicles and along the medullary cords, but in addition, it accumulates in the superficial cortical cell layers to form a rim around the node (Figure 54B).

The capacity of the phagocytes to localize antigen in the follicles is highly radioresistant and even at x-ray doses of 8,000 R there is still some capacity for follicular localization (Figure 55).

When the antigen is injected intravenously, much of it is retained in the spleen. Within 3 hours after injection, the label is almost all localized in the white pulp. It forms a heavy ring in the marginal zone which

is clearly separated from the heavily labeled germinal center (Figure 56). Two to three days after antigen injection, the label is found exclusively in the germinal center. Irradiation with 450 R one day prior to antigen injection did not affect the pattern of distribution, but the amount of antigen retained was greatly reduced. Radioactivity counts showed that less-than-normal amounts of label were initially retained in the spleens of the irradiated rats and that the rate of loss was more rapid. There was little or no antigen localization in the thymus.

The radiation-induced localization of antigen in the cortical layers of the node was similar with different preparations and doses of antigen; and procedures which restored antibody-forming capacity (see paragraph 1) did not in any detectable manner alter the radiation-induced changes.

CONCLUSIONS

Irradiation with 450 R one day before antigen injection decreased the capacity of the tissue to localize antigen in the lymph node follicles. Since follicular

localization has been demonstrated to play a role in determining the intensity of the immune response,⁽²⁾ we suggest that the observed effect of radiation on follicular localization is possibly one of the radiosensitive phases of immune response.

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TRITIATED THYMIDINE UPTAKE IN IRRADIATED RATS INJECTED WITH ANTIGEN

Bernard N. Jaroslow

PURPOSE AND METHODS

In studies of the radiation inhibition of antibody synthesis and the various ways by which this can be overcome, little attention has been paid to the cellular changes that occur following various restorative or stimulatory treatments (see review by Taliaferro, *et al.*⁽¹⁾). In this study, which is still in progress, I am investigating differences in tritiated thymidine uptake between irradiated and control rats and am comparing them to two groups of rats exposed to different restorative procedures, i.e., 1) irradiation 2 days after antigen injection and 2) the injection of colchicine and antigen at the same time, 1 day after irradiation.

Four groups of Wistar albino rats treated in the following manner were injected into the footpad with 10 μ g of flagella from *Salmonella adelaide*: 1) no further treatment; 2) 450 R one day before the injection of antigen; 3) 450 R one day before antigen injection and a subcutaneous injection of 200 μ g of colchicine at the same time as antigen; and 4) 450 R 2 days after the injection of antigen. On days 1, 4, 7, and 14 after antigen injection, three animals from each group were injected with tritiated thymidine and killed 1 hour later. The spleens, popliteal lymph nodes, and thymuses were sectioned and dipped in Kodak NTB-2 Nuclear Track

Emulsion to obtain autoradiographs. After 45 days, the slides were developed and stained with methyl green and pyronin.

I counted the number of labeled cells in the different regions of the tissue sections: in the lymph node, these were the follicle, the diffuse cortex and the medullary cords; in the spleen they were the white pulp and the red pulp; and in the thymus, the medulla and cortex were the specific regions counted.

PROGRESS REPORT

The early data indicate that there are more labeled cells in the white pulp of the spleen, the follicle of the lymph node and possibly in the cortex of the thymus of the irradiated non-antibody-forming group than in the three antibody-forming groups. Histologically, the two irradiated antibody-forming groups, i.e., those exposed to restorative procedures, were identical in the amount of cellular depletion, as was the irradiated control group. It is necessary, however, to confirm these preliminary findings before proceeding any further to an interpretation of these surprising results.

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OBSERVATIONS PERTINENT TO THE GENESIS OF HASSAL'S CORPUSCLES

Bernard N. Jaroslow

PURPOSE AND METHODS

The genesis of Hassal's corpuscles in the thymus, as postulated by Mackay *et al.*,⁽¹⁾ occurs in the following sequence: 1) the occlusion of capillary segments (most commonly in the medulla), 2) the inspissation of the contents, 3) the necrosis of the vascular endothelium, and 4) the stimulation of the proliferation of the neighboring endothelium. This results in the typical onion-like structure of the corpuscle. We have some evidence in support of the blood-vessel origin of Hassal's corpuscles.

Ten-week-old, Wistar albino rats were x-irradiated with 450 R and one day later they were injected with 10 μ g of I¹²⁵-labeled flagella prepared from *Salmonella adelaide* by standard procedures.⁽²⁻⁴⁾ They were sacrificed 2 days after antigen injection and autoradiographs of thymus sections were prepared by dipping them in Kodak NTB-2 Nuclear-Track Emulsion after which they were developed (after an appropriate exposure) and stained with pyronin and methyl green.

PROGRESS REPORT

In sections from both control and irradiated rats, 3 hours after antigen injection, the label was restricted to blood vessels. At 2 and 3 days after antigen injection, there was no detectable label in the thymus of any of the seven control rats that received labeled antigen; but in three of the seven rats that were irradiated with 450 R, the label was localized in Hassal's corpuscles (Figure 57A). In all rats exposed to radiation between 950 and 16,000 R (11 rats), and examined 1-2 days after antigen injection, the label was found both in Hassal's corpuscles and the blood vessels (Figure 57B) with a much greater frequency than in the 450 R irradiated rats.

CONCLUSIONS

From our findings, it appears that antigen persisting in the thymus is only that kept by entrapment when segments of small blood vessels become occluded. If there are few Hassal's corpuscles, and the rate of their development, by occlusion, inspissation, and epithelial proliferation⁽¹⁾ is slow, very little, but in some instances, detectable amounts of an antigen would be trapped in Hassal's corpuscles. This has been reported by Marshall and White⁽⁵⁾ and Gitlin *et al.*⁽⁶⁾ By our procedures, we did not observe any antigen in normal animals two days after antigen injection. If, however, the animal was irradiated with a small dose of x-ray

(450 R), antigen was found in easily detectable amounts in what are clearly Hassal's corpuscles, and in high-dose irradiated rats (950-16,000 R) the label was also found to be absorbed on the walls and in the lumens of small vessels.

These findings are closely correlated with the observations of Rhoades⁽⁷⁾ on vascular changes in irradiated animals. She observed that there were fewer small vessels in a section of irradiated skin than in normal skin. There was also a loss of collagen from the *tunica adventitia* of larger vessels and swelling of endothelial cells, followed by a variable amount of vascular occlusion.

Therefore, one might expect that a process such as described by Mackay *et al.*⁽¹⁾ would occur with increasing frequency as the x-ray dose increased.

FIG. 57.—A, section of rat thymus 3 days after irradiation with 450 R; note the heavy labeling in the Hassal's corpuscles (HC). B, section of rat thymus 3 days after irradiation with 8000 R; note the heavy labeling in the lumen of the vessels (V) and along the vessel walls.

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ANTIBODY SYNTHESIS IN ORGAN CULTURE

Bernard N. Jaroslow and Kenneth Shortman*

PURPOSE AND METHODS

Antibody formation *in vitro* offers an excellent system in which to study the direct pharmacological effects of drugs on antibody-forming cells. Actinomycin D has been found to inhibit antibody synthesis *in vitro* when present in the medium during the secondary or the primary response.⁽¹⁻³⁾ Smiley *et al.*⁽²⁾ found that the dose at which actinomycin D inhibited messenger-RNA synthesis was less (by a factor of 5), than that which inhibited antibody synthesis.

We initiated studies on actinomycin D inhibition of antibody synthesis *in vitro* as part of a program to use pharmacological agents of relatively specific antime-tabolite activity as a means for studying the sequence of biochemical steps leading to the synthesis of the antibody.

Wister albino rats were immunized by foot-pad injection with *Salmonella adelaide* flagella 2 to 6 months before sacrifice. The popliteal nodes were cut into 6-8 pieces and placed in Leighton tubes containing 1 ml of medium (Eagle's minimal essential medium plus 15% foetal calf serum). The secondary response was initiated according to the technique of Michaelides and Coons⁽⁴⁾ by the addition of a small amount of antigen (1 μ g of polymerized flagellin) and incubated at 37°C under an atmosphere of approximately 3% CO₂ and 97% air. The medium was completely removed and exchanged for fresh medium every three days; antibody titrations were performed by the bacterial immobilization test.

PROGRESS REPORT

The work performed to date was primarily aimed at stabilizing and characterizing the synthesis of antibody

in the organ culture system. We found that the substitution in the medium of hydrocortisone for serum, as recommended by Ambrose,⁽⁵⁾ was ineffective in our system. The induction of the secondary response was successful and the curve of antibody production was characterized by a 3-5 day latent period, with a peak titer at about day 10-11, and a decline to termination of production by the 20th day.

If antigen was added one day after initiation of the culture, the response was definite but small. Addition of antigen on the third day gave a barely detectable response and its addition on the seventh day gave a negative result. We found that as little as 0.1 μ g of polymerized flagellin added to the culture for 30 minutes prior to its being washed out induced a fairly strong response. Our standard antigen dose was 1 μ g polymerized flagellin for 24 hours on the day of culture, prior to its removal by replacement of the culture medium.

Actinomycin D was added for 4 hours on the eighth day of culture, i.e., during the phase of intense antibody synthesis and we observed the following: 10 μ g of actinomycin D stopped all antibody production; 1 μ g decreased it by about 95%, and 0.1 μ g barely depressed antibody output. The addition of 1 μ g actinomycin for 4 hours, prior to its removal, inhibited all antibody synthesis when it was added to the culture a) before the antigen, b) at the same time as the antigen, and c) 1 or 3 days after the addition of the antigen, i.e., during the latent period.

Actinomycin D (1 μ g/ml) irreversibly inhibits antibody synthesis. There is no apparent recovery after t^{11} free actinomycin D has been removed. The work being continued to determine the mechanism of antibody inhibition.

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TOTAL ANTIBODY—A PRACTICAL PARAMETER OF THE IMMUNE RESPONSE

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PURPOSE AND METHODS

An accepted measure of the intensity of the immune response is the peak titer of antibody. A more accurate measure of the response would be the total amount of antibody produced in response to an antigen. If the accumulative curve of the total antibody could be plotted, it would be useful in the construction of experimental tests of mathematical models of the kinetics and capacity of the mechanism of antibody formation.

The basic mathematics for the computation of total antibody was first presented by Taliaferro⁽¹⁾ in the following equation:

$$S_t = A_0 \frac{k_1 + k_2}{k_1} (e^{k_1 t} - 1)$$

where

S_t = total antibody

A_0 = serum antibody titer

t = time interval

k_1 = observed rate of antibody rise or decline in serum

k_2 = normal rate of decay of antibody (obtained experimentally by passive transfer of immune serum into a nonimmune host, followed by titration of recipient's serum to follow the rate of antibody disappearance).

The correction for partition of the antibody molecule in extracirculatory compartments is obtained from the data for the determination of k_2 .

To obtain valid information it is essential that the data refer only to a single class of antibody based on decay and partition.

The rabbit anti-Forsman hemolysin response is a system in which the response during the first 30 days appears to be almost pure $\gamma_1(\gamma_M)$ on electrophoretic separation and is 100% 2-mercaptoethanol sensitive.^(2, 3)

In the rat anti-*Salmonella* flagellin system, we utilize the 2-mercaptoethanol-resistant antibody titer, which appears to be $\gamma_2(\gamma_G)$ globulin.⁽⁴⁾

PROGRESS REPORT

The computation of total antibody synthesis can be done with a computer. The computer program we have created needs the following information:

- 1) serum antibody titer and date of sampling
- 2) rate of antibody decay
- 3) partition coefficient of the specific antibody.

With the above data, the program provides the following output for each experimental group. It calculates

- 1) mean geometric serum titer for each sampling
- 2) mean geometric peak titer for the group
- 3) length of the latent period
- 4) the number of days from the earliest detection of antibody to its peak titer in the serum
- 5) accumulated total antibody for any time during the sampling period
- 6) total antibody produced during the interval between any two samples
- 7) the amount of antibody synthesized on any given day
- 8) the average rate of antibody synthesis on any given day or over any given interval.

The output also provides, for measures 1 and 2, the number of animals in the group, variance, standard deviation and standard error of the mean. If an XY recorder is available, the program will plot outputs 1, 5, 7, and 9 on the same abscissa with the appropriate ordinates drawn in.

CONCLUSIONS

To date we are attempting to create a mathematical expression from the curve of total antibody in which the specific constants are obtained from a best fit of the data. On this basis, we could calculate the asymptote and values for the curve at any finite time. We hope to extrapolate these findings to create mathe-

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mathematical models of biological hypotheses which can then be experimentally tested.

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Theoretical Biology

DYNAMICS OF CELLULAR POPULATIONS

Ernesto Trucco and George A. Sacher

PURPOSE AND METHODS

The increasing number of experiments performed on cellular populations, both *in vivo* and *in vitro*, makes it desirable to have some mathematical framework for describing and understanding these phenomena. There are several approaches to this problem, ranging from the deterministic equations of classical population theory, used primarily in demography,⁽¹⁾ to the more sophisticated mathematical apparatus of stochastic population processes and Monte Carlo simulation.

PROGRESS REPORT

A simple mathematical model is provided by the differential equations for the total population size, such as Pearl and Verhulst's logistic equation. This has been used⁽²⁾ as a model for the recovery properties of cellular populations displaced from their normal state by the effect of radiation.

Another simple deterministic model is given by the Von Foerster equation which takes into account the age structure within the population. A brief summary on the meaning and scope of this equation is contained in last year's Annual Report.⁽³⁾ It will not be repeated here because full details can be found in three papers,⁽⁴⁻⁶⁾ of which one has recently been published and the other two will appear in print in the very near future. The following developments on applications of the Von Foerster equation have not been reported previously; they are treated at greater length in two papers:^(7, 8) 1) A problem on grain count halving in populations of labeled cells, previously discussed by S. A. Tyler and R. Baserga, can be solved and generalized with the help of Von Foerster's formalism. 2) The equations obtained by T. T. Puck in connection

with his work on the *collection function* have been derived from the age density function of an equivariant cellular population growing exponentially without losses from death or migration. Some minor errors that had crept into Puck's original analysis have been corrected.

CONCLUSIONS

At present we are considering the possibility of employing the more elaborate models provided by the stochastic theory; in particular, it would be interesting to determine whether the very general methods and results of J. E. Moyal⁽⁹⁻¹¹⁾ may be adapted for use in biological situations. Meanwhile, it is clear that only very simple problems can be solved analytically, so that future progress will have to come from computer calculations, such as the study of granulocyte turnover in the dog. (See the report on computer simulation of granulocyte kinetics.)

A different project involving machine computations has been started quite recently in collaboration with R. J. M. Fry, G. M. Kollmorgen, and M. D. MacLaren.* Here it is attempted to simulate by a Monte Carlo procedure the curves obtained experimentally for the percentage of mitoses that are labeled in a cellular population exposed to a pulse of tritiated thymidine at time zero. The simplest theory assumes that all the cells have identical generation times and that the population is not synchronized. The Monte Carlo calculation is designed to include the variability stemming from the distribution of life-cycle times (or from other sources, such as differences between animals), and the effects of synchronization. No r

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sults are as yet available, and full details on this study will be given in a later report.

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ON THE ESTIMATION OF SIZE DISTRIBUTION FROM PLANE SECTIONS

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PURPOSE AND METHODS

Estimating the distribution of sizes of particulate matter from plane sections has been a problem of great concern to research workers in many fields. In particular, the morphologist investigating the deposition of a class of substructures embedded in tissue from randomly chosen micrographs of sections would be concerned with size, shape, and uniformity of these bodies with respect to their original 3-dimensional orientation.^(1, 2) An example of the need for determining the distribution of sizes arises in morphological studies of adipose tissue. Quantitative methods are sought as bases for reliable interpretation of physiological changes that occur in fat cells with development and with advancing age. The size of the fat droplet is of great interest as one indicator of the metabolic activity of cells in a given depot. Physical measurements of fat droplets are taken from randomly chosen photographs of 10 μ microscopic sections of the epididymal fat pad of rats. In this note, we are primarily concerned with the construction of optimal statistical procedures relative to an idealized class of structures with the intent of establishing a methodology that will lend to further exploration and generalization.

PROGRESS REPORT

We envision the problem as follows. Given an opaque medium within which spheres of varying radii are randomly embedded (radii of the spheres are small rela-

tive to the dimensions of the cube). A planar cut is made and the frequency of circle radii in a section is tabulated. An estimate is desired of the size distributions of spheres in the cube.

Any estimation procedure must be based on the relationship between the distribution of sphere radii $Q(R)$ and section circle radii $P(r)$. In previous investigations⁽³⁻⁵⁾ this relationship has been derived either for the case in which

- 1) $Q(R)$ is a discrete distribution on a finite sample space, or
- 2) $Q(R)$ is differentiable.

In this study, an integrated theory is developed. Under the assumption of zero thickness for the section $P(r)$ is derived in terms of $Q(R)$ in the most general form, and in particular when $Q(R)$ is absolutely continuous with respect to some measure μ (case 1 and 2 being special cases). Estimation procedures to suit various special cases are considered next. If μ is the counting measure on a finite sample (i.e., case 1), it is shown that, in general, estimates can be determined by least squares procedures. If μ is the Lebesgue measure over the positive real numbers (covering, of course, case 2), then first required is the solution of the integral equation

$$g(r) = 2r \int_r^\infty \frac{G(R)dR}{\sqrt{R^2 - r^2}}, \quad r > 0, \quad (1)$$

where $G(R) = N$ times the density of $Q(R)$, N = total number of spheres in the given cube (assumed, without loss of generality, to be of unit volume), and $g(r) = \int_0^\infty 2R G(R)dR$ times the density of $P(r)$. $\varphi(t) =$

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$\int_t^\infty G(R)dR$ = number of spheres of radius greater than t , is obtained explicitly in terms of $g(r)$ as follows.

$$\varphi(t) = \frac{1}{\pi} \int_t^\infty \frac{g(r)dr}{\sqrt{r^2 - t^2}} \quad \text{for all } t \text{ such}$$

that $0 < t < \infty$. This leads to an unbiased estimate

$$\hat{\varphi}(t) = \frac{1}{\pi} \sum_{r>t} \frac{1}{\sqrt{r^2 - t^2}}, \quad (2)$$

where r now stands for the observed circle radii. Under very general nonparametric assumptions, $\hat{\varphi}(t)$ is shown to be the unique minimum variance "conditionally" unbiased estimate. An estimate for the standard error of $\hat{\varphi}(t)$ is also provided.

An attempt is made to extend the procedure to the case where section thickness is any arbitrary number δ .

CONCLUSIONS

The proposed method overcomes the drawbacks of the method of Bach⁽⁵⁾ which approximates the integral in (1) from zero to t by substituting for the integral summations involving arbitrarily chosen points $x_j (j = 1 \cdots n; x_j \geq 0)$ and solving for estimated number of spheres of radius between x_{j-1} and x_j , recursively. Three deficiencies are inherent in Bach's procedure:

1) different numerical answers result from different choices of x_j ; 2) the estimate is biased; and 3) it constitutes a summation of errors due to recursive calculations.

The techniques developed may be applicable to the situation in which sampling is performed from a mixture of populations. For example, suppose $\chi(x, a)$ is a c.d.f. that depends on a parameter a which, in turn, has a c.d.f. $U(a)$. We desire to estimate the mixing distribution $U(a)$, given samples from $V(x) = \int \chi(x, a)dU(a)$.

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ON A MINIMIZATION PROBLEM

Lalitha Samalhanan* and Sylvanus A. Tyler

PURPOSE AND METHODS

Srikantan⁽¹⁾ gave an iterative process for obtaining, in a stratified sampling design, optimum allocation of the total sample size to the various strata with minimum variance at specified cost, while restricting the strata sample sizes. He then generalized this method by determining an iterative solution to the problem of minimizing an additive convex function of n variables, i.e., a function of the form

$$\varphi(x_{1,1}) \cdots x_{n,1}) = \sum_{i=1}^n \varphi_i(x_{i,1}) \quad (1)$$

where $\varphi_i(x_{i,1})$ is a twice differentiable, strictly convex function of the single variable $x_{i,1}$ and the $x_{i,1}$'s are constrained by

$$\sum_{i=1}^n c_i x_{i,1} = C \quad (c_i \neq 0) \quad (2)$$

$$a_{i,1} \leq x_{i,1} \leq b_{i,1}; \quad i = 1 \cdots n \quad (3)$$

($a_{i,1}$ can be $-\infty$ and $b_{i,1}$ can be $+\infty$)

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Proof of optimality was based on the Kuhn-Tucker theorem.⁽²⁾

This problem was considered by the first author while at the University of Chicago, and an approach was developed that involves the solution of a finite sequence of similar problems free of restriction (3). With this approach, the method of Lagrange multipliers could be utilized. The solution was further simplified computationally.

PROGRESS REPORT

This summer the above approach was extended to the more general problem of minimizing (1) subject to (2) and the sequence of constraints

$$a_{i,j} \leq x_{i,j} \leq b_{i,j}; \quad \left. \begin{matrix} i = 1 \cdots n_j \\ j = 1 \cdots r \end{matrix} \right\} \quad (4)$$

where

$$x_{i,2} = \sum_{k \in F_{i,2}} c_k x_{k,1}; \quad F_{i,2} (i = 1 \cdots n_2)$$

are disjoint sets and for each $j = 3 \cdots r$

$$x_{i,j} = \sum_{k \in F_{i,j}} c_k x_{k,j-1}; \quad F_{i,j} (i = 1 \cdots n_j)$$

are disjoint sets. This situation can occur, for instance, in capital budgeting in an organization that has a hierarchy. Maximization of total profit represented by an additive concave function (equivalent to minimizing its negative) is desired, subject to an over-all budget constraint plus bounds at each step in the hierarchy. (See diagram below.)

A similar procedure could also be used in some experimental designs for large and/or long-term animal studies where costs are directly proportional to the number of animals utilized at various levels.

CONCLUSIONS

As pointed out by Srikantan,⁽¹⁾ the constraining equality need not be of the type $\sum c_i x_i = C$. It is sometimes possible to convert an equation by suitable transformation to the above form with transforms satisfying the other requirements. An example of this is the problem of minimizing cost in a stratified sampling scheme, while holding the variance of the global estimate at a specified level and subjecting strata sample sizes to minimum limits. This is equivalent to minimizing $\sum c_i x_i + K$ subject to $\sum_{i=1}^n d_i/x_i = C$ and $a_i \leq x_i (i = 1 \dots n)$ where $x_i = i^{\text{th}}$ stratum sample size, $c_i =$ cost per unit for the i^{th} stratum, $K =$ combined overhead costs, $d_i = N_i/\sigma_i^2$ ($N_i = i^{\text{th}}$ stratum size and $\sigma_i^2 =$ variance of the i^{th} stratum), and $C =$ the prescribed total variance. Further discussion and additional examples can be found in Reference 1.

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STOCHASTIC THEORY OF MORTALITY

George A. Sacher, Ernesto Trucco and David A. Woodward*

PURPOSE AND METHODS

The main purpose of this project is to study the stochastic aspects inherently connected with the occurrence of mortality in large populations. As pointed out in previous reports, it has been found necessary to perform the computations by means of a Monte Carlo program after all feasible approaches of a purely analytic nature had been exhausted. The model chosen for this investigation is provided by an ensemble of particles in Brownian motion inside a potential well, with escape of a particle from the well corresponding to death of an individual member of the population.

PROGRESS REPORT

The first phase of the program is now completed; some preliminary results have been obtained although not yet analyzed. This part of the program is designed to solve the stochastic differential equation (or Lange-equation) $\Delta x/\Delta t = \beta x + F(y) + \sigma A(t)$, where $\Delta x = dy/dt$, $F(y)$ is a quadratic function of y , i.e., $F(y) = ay^2 + by + c$ (with a , b , and c constants),

σ is a positive constant, and $A(t)$ is a Gaussian random disturbance (white noise) with unit variance and mean zero. Thus, x corresponds to the velocity and y to the position of a particle in the potential well. $F(y)$, the restoring force is derived from a cubic potential function, $V(y) = -(a/3)y^3 - (b/2)y^2 - cy + \text{constant}$.

In contrast to previous attempts, the present program computes the path of each particle by means of a second order finite difference approximation. Besides a , b , c , and σ , the initial distributions of particle positions and velocities must be given as input variables. For better statistics in evaluating the escape probabilities, the program uses a forcing procedure (i.e., an appropriate modification of the potential function; see Reference 1). The output includes an estimate of the probability that a particle will leave the well; moreover the two first moments and the three second moments for the joint distribution of particle position and velocity are estimated, based on the population of particles remaining inside the well at the end of the trial period. It now appears that the calculation of escape probabilities is quite reliable, but the estimation of the moments still requires some improvement.

* Applied Mathematics Division.

Another important aspect of the theory has been dealt with in a paper recently published by one of us.⁽²⁾ It is shown that the stochastic nature of the mortality process has far-reaching consequences for interpreting the lethality *vs.* total dose curves that are obtained by administering whole-body irradiation to groups of mice.

CONCLUSIONS

Work on the Monte Carlo program still constitutes our primary concern and will be continued during the next fiscal year. The over-all, long-range objective of this project, namely, a fully integrated theory stressing

the stochastic and the system-theoretic aspects of the life and death process, remains unchanged.

The task of setting up the Monte Carlo program was handled with the technical assistance of Miss Janet Herman.

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SPECIFICATION AND QUANTITATION OF THE CONSTITUENCY IN A RADIOACTIVE MIXTURE FROM COUNT-RATE DATA

Sylvanus A. Tyler, Merlin H. Dipert, William D. Fairman and Jacob Sedlet**

PURPOSE AND METHODS

Of constant concern to both the layman and the scientist is the biological effect of radionuclides naturally present† or recently introduced‡ into the air we breathe,⁽¹⁾ the soil we till,⁽²⁾ and the food we eat.⁽³⁾ This omnipresent burden we bear consists of a mixture of parent substances and their daughter products that are constantly changing in concentration. Assessment of this burden requires the detection, identification, and resolution of individual nuclides from among the mixture. Many mathematical techniques, statistical decision procedures, and computing schemes to aid in the task have been announced;⁽⁴⁾ most are based on the a priori assumption that the number and identity of the constituent nuclides are known.

PROGRESS REPORT

We have constructed a procedure⁽⁵⁾ based on the more realistic and less stringent assumption that the nuclides actually present in the mixture are included in a selected list of candidates. Compilation of such a list is usually not difficult in that indicative information (such as the origin and history of the mixture, the separation chemistry, and counting techniques), that suggests some near-minimal set of nuclides for inclusion is frequently available.

Suppose the total count-rate C_i from a mixture of

radionuclides is recorded at various times t_i . These times are measured in relation to zero time at which it is assumed that the mixture is composed solely of parent substances. The composition of the mixture is unknown except that the components are among a known list of radionuclides for which the decay chain and associated decay constants and detection efficiencies of each member are given. The problem is to 1) identify the parent substances in the mixture, 2) determine the proportion of each in the mixture, and 3) estimate the contribution of each to the total count. By use of the method of least squares, an algorithm has been constructed that achieves the desired solution when the list includes nuclides with nonbranching (1) and/or branching (2) decay chains:

A two-part computer program in Fortran (see Figures 58-60) was also constructed to facilitate computations and satisfy storage limitations of a medium-speed electronic computer.

CONCLUSIONS

The assumption of a known zero time still restricts the applicability of this method to the case where the

* Industrial Hygiene and Safety Division.

† Cosmic-ray produced radionuclides in the atmosphere and natural radioactivity in the earth.

‡ Fallout and radiation due to industrial, technological, and medical uses of radioactive sources.

FIG. 58—Determination of Components of Radioactive Decay Mixtures—CORD—Part I

* See Figure 60 for details of computation of RHO (J + 1)

FIG. 59—Determination of Components of Radioactive Decay Mixtures—CORD—Part II

FIG. 60—Computation of $RHO(J+1)$ in Figure 58

time of creation (as in activation analysis) or separation time is experimentally determined or where only stable nuclides result from decay of the parent radionuclides. Attention is being directed toward evolving a more general solution free of this limitation.

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COMPUTER SIMULATION OF GRANULOCYTE KINETICS

Ernesto Trucco, Robert N. Buchal,* and Mary Maloney†

PURPOSE AND METHODS

We desire to set up and solve simple equations describing the behavior of the last four stages in the granulocyte population. These stages include the non-proliferative part of the total life-span; they are: a) metamyelocytes, b) band cells, c) mature polymorphonuclear granulocytes, all of them in the marrow, and d) mature polys in the blood. The experiments of H. M. Patt and M. A. Maloney⁽¹⁻³⁾ were performed on dogs (beagles) by labeling the cells *in vitro*, generally with a single injection of tritiated thymidine (H^3T). The label is taken up only by dividing cells and is subsequently kept within the maturing and functional cells derived from them. In a labeled cohort of cells, the fluxes are not constant in time, but the total population, consisting of labeled and unlabeled cells, is assumed to be in a steady state.

Each of the above stages (also called "demes") is represented by one Von Foerster equation, as explained in last year's Annual Report [see also Reference 4]. The dependent variable in this equation is the age density function which, when integrated over the age variable, gives the total expected number of cells with a specified grain count, as a function of time.

In order to obtain a complete description of the system it is necessary to make assumptions about the *loss function* for each deme; this indicates the age dependence of cell death or of emigration to a subsequent deme. Moreover, the metamyelocyte input probability (which describes the flow of labeled cells into the metamyelocyte stage) must also be given or inferred from other experimental data.

Independent of any specific assumption on the nature of input flow, death, or migration, the Von Foerster equations show that the total system should obey the *area rule* which represents a conservation law for the amount of label being transferred from deme to deme after the initial injection of H^3T .

PROGRESS REPORT

Preliminary studies showed that finding the correct form of the metamyelocyte input function constitutes a major problem. By trial and error, one such function has been constructed that may be acceptable, or at least consistent with the experimental results. This,

however, has some consequences and implications for the *myelocyte* population which have not been fully explored. Instead of limiting the investigation to the nonproliferative part of the system, we are led to speculate on cellular dynamics and self-renewal properties of the myelocyte deme (the last stage of the *dividing* cells). It now appears that the myelocytes provide an input of labeled metamyelocytes which is larger and lasts for a much longer time than one would anticipate from simple considerations of grain-count halving with each generation of metamyelocytes. It may be possible to account for this effect by assuming that *unlabeled* myelocytes have a large probability of dying (more precisely, that healthy and well-nourished cells also have a better opportunity of taking up the tritium label). However, a satisfactory formulation of such a model, i.e., one that would agree with the area rule, has not been found.

A similar difficulty is encountered at the marrow-blood barrier, where again it was found experimentally that the total number of labeled polys entering the circulation is larger than would be predicted from the area rule. At present, it is not clear whether these discrepancies must be attributed to some inadequacy of the model or to experimental error, although the latter possibility seems unlikely.

One further question that was examined concerns the nature of the band-cell deme. It had previously been stated (e.g., in Reference 3, end of p. 521) that the band cells obey "first in—first out" kinetics. The computer results indicate that this is not necessarily true and that, in fact, the band cells might equally well emigrate "at random." On this point, further experiments are needed to clarify the situation.

CONCLUSION

The main conclusion at this time is that a great deal of work, both theoretical and experimental, still remains to be done in order to understand clearly the properties of neutrophil granulocytes, as they evolve from blast cells to mature polys.

The computer program runs very smoothly and has given excellent results.

The present lines of investigation will be continued during the next fiscal year.

We are grateful to A. J. Strecok of the Applied Mathematics Division for writing and operating the computer program which is being used in performing the calculations.

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RATE OF CONVERGENCE IN THE EMPIRICAL BAYES APPROACH TO THE TWO-ACTION PROBLEM

John Van Ryzin,[†] Lalitha Sanathanan, and Sylvanus A. Tyler

PURPOSE AND METHODS

Robbins⁽¹⁾ and Samuel⁽²⁾ deal with the application of the empirical Bayes approach to the two-action problem (cf. Section 3 of Reference 1 and Section 4 of Reference 2). The notation and some results in Reference 1 are used here.

We refer to Theorem 2 in Reference 1, where an asymptotically optimal sequential decision rule is given.

The purpose of this note is to show that if $\phi_{n+1}(x_1 \cdots x_n; x)$ is chosen to be the average of n independent identically distributed random variables

$$h_i(x) \quad (i = 1 \cdots n)$$

such that

$$(a) \quad E[h(x)] = \phi_G(x)$$

and

$$(b) \quad \int_X \sqrt{\text{var } h_1(x)} d\mu(x) < \infty,$$

then

$$R(t_{n+1}^*, G) - R(G) = O(n^{-1/2}) \quad \text{as } n \rightarrow \infty.$$

where

$$t_n^* = t_n^*(x_1, \cdots, x_n) = \begin{cases} 1 & \text{if } \phi_n(x_1, \cdots, x_{n-1}; x_n) \geq 0. \\ 0 & \text{otherwise} \end{cases}$$

PROGRESS REPORT

Two proofs (the first due to first author and the second due to second author) are given below.

The following results from Reference 1 are required:

$$R(G) = L_0(G) - \int_X t_G(x) \phi_G(x) d\mu(x) \quad (1)$$

[†] Applied Mathematics Division.

$$R(t_{n+1}^*, G) = E[L_{t_{n+1}^*}(\lambda_{n+1})] \\ = L_0(G) - E[t_{n+1}^* b(\lambda_{n+1})]. \quad (2)$$

$$E[t_{n+1}^* b(\lambda_{n+1})] = \int_X \left[\int_X \cdots \int_X t_{n+1}^*(x_1 \cdots x_n; x) f_G(x_1) \cdots f_G(x_n) d\mu^n \right] \phi_G(x) d\mu(x). \quad (3)$$

Or

$$E[t_{n+1}^* b(\lambda_{n+1})] = \int_X \{P[\phi_{n+1}(x_1 \cdots x_n; x) \geq 0] \phi_G(x)\} d\mu(x). \quad (4)$$

Proof 1

From (1), (2), and (3),

$$R(t_{n+1}^*, G) - R(G) = E \left[\int_X \{t_G(x) - t_{n+1}^*(x_1 \cdots x_n; x)\} \phi_G(x) d\mu(x) \right] \quad (5)$$

where E refers to expectation w. r. t. joint distribution of $x_1, x_2, \cdots, x_n$. Expression (5) can be rewritten as

$$E \left[\int_X \{t_G(x) - t_{n+1}^*(x_1 \cdots x_n; x)\} \phi_G(x) - \phi_{n+1}(x_1 \cdots x_n; x) \} d\mu(x) \right] \\ + E \left[\int_X \{t_G(x) - t_{n+1}^*(x_1 \cdots x_n; x)\} \phi_{n+1}(x_1 \cdots x_n; x) d\mu(x) \right]. \quad (6)$$

However, the second term in (6) is ≤ 0 , since $t = t_{n+1}^*$ minimizes the expression

$$\int_X t \phi_{n+1} d\mu(x) \quad \text{conditionally on } x_1 \cdots x_n.$$

Since $t_{n+1}^*(x_1 \cdots x_n; x)$ and $t_G(x)$ are 0, 1 functions, (5) is bounded by

$$E \left[\int_X |\phi_G(x) - \phi_{n+1}(x_1 \cdots x_n; x)| d\mu(x) \right] \\ = \int_X E[|\phi_{n+1}(x_1 \cdots x_n; x) - \phi_G(x)|] d\mu(x) \quad (7)$$

$$\leq \int_X \sqrt{E[\{\phi_{n+1}(x_1 \cdots x_n; x) - \phi_G(x)\}^2]} d\mu(x)$$

by Schwarz inequality

$$= \int_X \sqrt{\text{var } \phi_{n+1}(x_1 \cdots x_n; x)} d\mu(x) \\ = \frac{1}{n^{1/2}} \int_X \sqrt{\text{var } h_1(x)} d\mu(x).$$

This completes the proof.

Proof 2

By (1), (2), and (4)

$$R(t_{n+1}^*, G) - R(G) = \int_X \{[\phi_G(x)]^+ \\ - P[\phi_{n+1}(x_1 \cdots x_n; x) \geq 0] \phi_G(x)\} d\mu(x) \quad (8)$$

If $\phi_G(x) = 0$, then the integrand in (8) is zero.

$$\int_X \sqrt{\text{var } h_1(x)} d\mu(x) = \sum_{x=1}^{\infty} \sqrt{\text{var } h_1(x)} = \sum_{x=1}^{\infty} \sqrt{E(h_1(x) - \phi_G(x))^2} \\ = \sum_{x=1}^{\infty} \sqrt{[x+1 - \phi_G(x)]^2 P[x_1 = x+1] + [-\lambda^* - \phi_G(x)]^2 P[x_1 = x] + \phi_G^2(x) P[x_1 \neq x]} \\ \leq \sum_{x=1}^{\infty} \sqrt{[x+1 + |\phi_G(x)|]^2 P[x_1 = x+1] + (\lambda^*)^2 P[x_1 = x] + 2|\lambda^*| |\phi_G(x)| + 2\phi_G^2(x)} \quad (10) \\ \leq \sum_{x=1}^{\infty} \sqrt{[x+1 + \sum_{x=1}^{\infty} |\phi_G(x)|]^2 P[x_1 = x+1] + \sum_{x=1}^{\infty} (\lambda^*)^2 P[x_1 = x]} \\ + \sum_{x=1}^{\infty} 2\sqrt{|\lambda^*| |\phi_G(x)|} + \sum_{x=1}^{\infty} 2|\phi_G(x)|.$$

If $\phi_G(x) > 0$, then it is equal to

$$\{1 - P[\phi_{n+1}(x_1 \cdots x_n; x) \geq 0]\} \phi_G(x) \\ = P[\phi_{n+1}(x_1 \cdots x_n; x) < 0] \phi_G(x) \\ = P[-\phi_{n+1}(x_1 \cdots x_n; x) + \phi_G(x) \\ > \phi_G(x)] \phi_G(x) \\ \leq P[|\phi_{n+1}(x_1 \cdots x_n; x) - \phi_G(x)| \\ > \phi_G(x)] \phi_G(x) \quad (9) \\ \leq \frac{E[|\phi_{n+1}(x_1 \cdots x_n; x) - \phi_G(x)|]}{\phi_G(x)} \cdot \phi_G(x)$$

(by Markov's inequality, since $\phi_G(x) > 0$)

$$E[|\phi_{n+1}(x_1 \cdots x_n; x) - \phi_G(x)|]$$

Similarly, if $\phi_G(x) < 0$, (9) is true.

Therefore we have (7) above and hence the final result. Suppose that instead of $t_G(x)$, we have $t_K(x)$, obtained by replacing $\phi_G(x)$ by an arbitrary function $K(x)$ and we use $t_{n+1}^K(x_1 \cdots x_n; x)$ as approximation to $t_K(x)$, where

$$t_{n+1}^K(x_1 \cdots x_n; x) = 1 \quad \text{if } K_{n+1}(x_1 \cdots x_n; x) > 0 \\ = 0 \quad \text{otherwise}$$

and $K_{n+1}(x_1 \cdots x_n; x)$ satisfies conditions analogous to (a) and (b). Then it follows from the above that if

$$\left| \frac{\phi_G(x)}{K(x)} \right| \leq C \quad \text{for almost all } x \text{ where } C \text{ is any constant,}$$

then

$$|R(t_{n+1}^K, G) - R(t_K, G)| = O(n^{-1/2}) \quad \text{as } n \rightarrow \infty.$$

Example.—Let us take the case of the Poisson distribution discussed in Section 4 of Reference 1. We can then define

$$h_1(x) = \begin{cases} x+1 & \text{if } x_1 = x+1 \\ -\lambda^* & \text{if } x_1 = x \\ 0 & \text{otherwise.} \end{cases}$$

Then $h_1(x)$ satisfies (a).

Also

But

$$\sum_{x=1}^{\infty} |\phi_G(x)| < \infty \quad \text{by (48) of Section 3, Reference 1.}$$

Therefore, from (10) it is clear that if

$$\sum_{x=1}^{\infty} (x+1) \sqrt{P[x_1 = x+1]} < \infty$$

and if

$$\sum_{x=1}^{\infty} \sqrt{\phi_G(x)} < \infty$$

then condition (b) is satisfied.

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A SYSTEM OF COMPUTER PROGRAMS TO RETRIEVE AND DISPLAY A LARGE QUANTITY OF BIOLOGICAL DATA AS AN AID TO ANALYSIS

Jeanne A. Pagels and Merlin H. Dipert

PURPOSE AND METHODS

The production of sizable quantities of data demands a systematic procedure for their acquisition and analysis. The application of computer methods, with the properties of speed and accuracy, are most desirable qualities that help solve these needs of the biologist. The objective of this paper is to illustrate the use of computer techniques in the retrieval and systematic display of large quantities of biological data.

A data base of qualitative and quantitative information that describes a large dog colony, as well as the necropsy records and weights of body parts of the dogs upon death, was given to us with a request to retrieve and organize this information as an aid in determining the cause of death. The computer languages, FORTRAN and VFAP, in conjunction with the FORWARD SORT/MERGE GENERATOR of the GE 225 medium-sized computer (four tape drives) were used to write the computer programs.

PROGRESS REPORT

When this project was suggested, the life histories of the animals were written on a single magnetic tape; whereas, the necropsy records and weights of body parts were on punched cards. To create a homogeneous system with comprehensive search capabilities, it was necessary to write a program which would assemble and update the necropsy records and weights of body parts on individual magnetic tapes. (In this paper and in Figure 61 the life-histories-data tape, necropsy-data tape, and weights-of-body-parts data tape will be referred to as "census" or "census tape," "necropsy tape," and "body-parts tape" respectively.)

After each dead animal number was obtained from the necropsy tape, the census tape was searched to select data relevant to the cause of death. This information consisted of both alphabetic and numeric characters with periods of time for two given dates calculated by the subroutine EPOCH. In order to group the animals with like life-histories, an internal sort was performed on subsets of each animal's data that yielded a numerically-ordered or alphabetically-ordered group depending on the type of data in each set. Once the data were prepared, the arrangement of variables written on tape demanded a definite sequence to satisfy future plans for categorizing the animals. Finally, a selected life-histories tape was obtained. This tape will be called SLH tape.

Following the assembly of the two data tapes, necropsy and SLH, (each in animal number order) a complete printout was executed that displayed a unified body of information within each animal and within the entire report. Because of the variable combinations in which the printed lines could appear for presentation of readable output, the memory of the computer was largely consumed by PRINT and FORMAT statements.

Because the SLH tape was in the proper order for grouping animals with identical life histories, the FORWARD SORT/MERGE GENERATOR was prepared next. This procedure consists of describing on punched cards the set of parameters to be sorted and merged. In a very short time, compared to hand sorting, a sorted data tape (designated SSLH tape) was obtained.

With the sorted information in hand, the technique of merging two sets of information (SSLH data with the necropsy or weights of body parts data) was undertaken. The speed of the merge was greatly reduced because the SSLH tape was no longer in animal number order. As the data were read from the SSLH tape for a particular animal, its necropsy or body-

FIG. 61.—Program design

part weights had to be searched on the other appropriate tape; this resulted in a delay. All the data were not combined on one tape because of the restrictions of the FORWARD SORT/MERGE GENERATOR in handling a limited number of parameters and record lengths. See Figure 61 for a scheme of the entire project.

COMPUTER SCIENCE

Merlin H. Dipert, Jeanne A. Pagols, and Carol A. Vancina

PURPOSE AND METHODS

The two main objectives of the computer science group are 1) the efficient operation of the machine complement to handle the biological and medical data effectively and 2) the design of effective statistical, mathematical, and data-systems routines necessary to store, retrieve, and analyze the scientific data so that the computer becomes an integrated tool in biological research.

To accomplish this, we maintain a GE 225 computer with 8192 twenty-bit words, a high-speed printer, four tape drives, an auxiliary arithmetic unit, and an on-line Cal Comp plotter.

The facilities and personnel of the Applied Mathematics Division are used to complement our own.

The basic concept of our system is a coordinated data base and a unified programming system for addressing the base. The system must also be capable of solving mathematical and statistical problems arising outside the base.

PROGRESS REPORT

A library is maintained of statistical and special routines. These are listed by title:

Statistical Routines

- 100 Cumulative percents (probit) least square with weighing factors and 10, 20, . . . 90 centile points
- 101 Means and variances, one at a time or N at a time
- 103 Decomposition of radionuclides
- 104 Polynomial regression—calculates theoretical points and will compute an orthogonal set if desired
- 105 Paired means and variances
- 106 Gompertzian $W = W_0 e^{A/(1-e^{-at})}$
- 107 Least Squares $S = 1 - (1 - e^{-\kappa D})^n$ —"Multi-hit"
- 108 Correlation coefficient

CONCLUSION

The advantage of computer methods to organize and display large quantities of data has been illustrated by the results of this system of programs now in use in the Division. Installations with similar or larger-scale facilities can adapt the basic design of this project with little difficulty.

- 109 Linear regression with confidence limits—computes calculated points
- 111 Analysis of variance—hierarchical design
- 112 Computation of radionuclide retention (half-life)
- 114 One-way analysis of variance with means and t test
- 115 Linear regression with two slopes
- 117 Eigenvalues and eigenvectors
- 118 Fisher's exact method
- 119 Simultaneous equations
- 120 Logistic
- 121 Multi-variate analysis of variance
- 125 Coulter Counter Program
- 126 EPOCH (Time between dates subroutine)
- 127 Normal probability frequency distribution, 1 to 3 curves
- 129 t test for slopes and intercepts
- 130 Plotter programs
- 132 Cross-section cell distribution—Schwartz method
- 133 Analysis of variance—three-way with proportionate subclasses
- 134 Analysis of variance— N -way with equal subclasses
- 135 Time series analysis, power spectrum, and plot
- 136 Two-way analysis of variance with Studentized Range Test

Special Programs

- 500 Personnel records
- 501 Rodent colony update and census
- 509 Chick protein calculation
- 510 Creates copies and updates dog tape
- 511 Radiation count—dog
- 513 Cause of death—dog
- 515 Dog tape sort
- 516 Dog census listing
- 519 Red Cell—mean volume
- 520 Bibliography

525 Calculate and plot food intake from semisynthetic diet—dog
 528 Dog litter history
 530 Red blood cell correction and listing
 533 Tricarb calculations—double tracer
 535 XAM-3 systems chicks
 537 Sterol metabolism data storage and retrieval—plot
 541 Analysis of port-a-punch cards
 542 Brain weight computation
 543 Guppy survival
 548 Sum of exponentials
 549 Colorado birth weight analysis

551 Correction factor and calculations on irradiated tissue
 555 Dog weight, least squares Gompertzian, plot
 557 Mother-daughter cell rates
 558 History of kennel occupancy
 559 Prepares, sorts, and assembles dog records
 561 Determination of instrument factors
 562 Frequency distribution of dog ages

Data Storage

Several data files are maintained with the larger ones on tape and the smaller ones on cards.

1 Environmental radiation (Gustafson)

FIG. 62.—Subroutine EPOCH to compute elapsed time between two dates

2. Mouse radiation genetics (Grahn)
3. Acute radiation effects—growth (Stearner)
4. Dog program (Norris)
5. Bibliography (Wolfgang)
6. Sterol metabolism (Klein)
7. Red cell life-span—guppy radiation—behavior studies (Sacher)
8. Tumor studies (Auerbach, Brues)
9. Rodent colony (Flynn)
10. Colorado birth weights (Auerbach, Grahn)

Special Interest

In addition to projects listed in other sections of this report, these were of special interest:

1) Dog program (Norris)—Several programs, which retrieve information from the data tape have been written. They are designed to compute and plot actual age-weight points and theoretical curves; calculate and plot semisynthetic food intake; and display in tabular form the litter history of dogs in breeding. Another program sorts and displays the output of radionuclide calculations. These programs were outlined at the Thirteenth Annual Meeting of the Radiation Research Society.

2) Rodent colony (Flynn)—The program reported in the last annual report is operating and weekly weaning schedules and mortality summaries are being provided.

3) In connection with Dr. Klein's work, a program has been written to calculate maximum instrument discrimination and progress has been made to plot the contour field.

4) In cooperation with the Applied Mathematics Division, a study is being made of the problems of time sharing, direct data input, and remote access to

the computer. To gain familiarity with some of the aspects of remote control and time sharing, two programs (reported in another section of this report) were written on a GE 235 in Phoenix, Arizona, utilizing a teletype in the Applied Mathematics Division.

5) Days between two dates subroutine (Program No. 126 EPOCH)—A subroutine was written to compute the number of days between two dates since this is the basic time unit in most experiments. The block diagram (Figure 62) illustrates the logic which will compute the time between any two dates in the Gregorian calendar.

6) The number of scientists who do their own programming has increased and we continue to assist them as requested.

Summer Program

Two student associates were with the Computer Section during the summer of 1965.

1) Mr. Ronald Rau (Duke University) wrote a program to compute inbreeding coefficients of the dog colony and also a program to compute energy constants for chemical elements. The last program was on the AMD-GE time-sharing system.

2) Mr. Buford Kirkwood (University of Illinois) wrote several computer programs: Size distribution of cells using the Schwartz method, three-way analysis of variance with proportionate subclasses, and a time series (power spectrum) analysis and plot. The program for the Schwartz method was coded in FORTRAN and BASIC for the time sharing system.

We wish to acknowledge the continuing cooperation of Mr. Sylvanus A. Tyler in program planning and mathematical analysis.

Laboratory Animal Medicine

LABORATORY ANIMAL MEDICINE. STATEMENT OF PROGRAM OBJECTIVES

Robert J. Flynn

The basic responsibility of the Research-animal Group is to provide and maintain the animals needed for the Division's research programs. Because little objective, experimentally-obtained information is available on the proper care of laboratory animals and the control of laboratory animal disease, the Research-animal Group, in addition to its service responsibilities, also studies the care, management and diseases of laboratory animals. Some of the research is aimed at solving operational problems. This is il-

lustrated by the accompanying reports of the status of the rodent breeding, disease control, and animal vital statistics computer programs. A large effort is devoted to studying laboratory animal diseases, particularly those that interfere with the Division's research program. This is illustrated by the accompanying reports on the disease status of commercial murine pneumonia, *Citrobacter freundii* infection, the intestinal microflora of mice and the fluorescent antibody diagnostic technic.

The Research-animal Group also provides cooperative, specialized veterinary medical and animal technologic assistance to the Division's research program. This is illustrated by reports to be found elsewhere in this publication such as one on the characterization by members of the Research-animal Group of a disease of ground squirrels that was interfering with the studies of the Physiology Group (see the section on Physiological Effects); and the reports of the professional assistance (dog breeding, roentgenography, surgery, microbiology and pathology) provided by the members of the Research-animal Group to the group concerned with the metabolism of fission products (see Toxicity and Metabolism of Radionuclides).

The number and kind of animals maintained in the Division's facilities and a listing of those acquired and those produced during the past year is shown in Table 34.

TABLE 34. ANIMAL INVENTORY

Species	Inventory as of Sept. 1, 1965	Total acquired Sept. 1, 1964-Sept. 1, 1965	Total produced at Argonne Sept. 1, 1964-Sept. 1, 1965
Canine			
Beagles	633	0	129
German shorthair pointers	28	0	0
Mongrels	3	0	0
Mice			
Germfree	28	199	50
SPF	9,447	0	44,687
Conventional	13,898	2,692	5,215
Rats	4,048	1,345	7,141
Guinea pigs	32	360	0
Rabbits	118	223	0
Chinchillas	29	0	0
Ground squirrels	140	300	0
Chinese hamsters	11	4	7
Wild mammals (several species, mostly rodents)	1,118	42	
Monkey	1	0	0
Chickens	440	6,900	0
Frogs	0	631	0
Axolotls	56	86	0
Fish	46	0	
Snails	60	0	0
Grasshoppers	900	100	

RODENT BREEDING PROGRAM

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

PURPOSE AND METHODS

This program was initiated to provide more highly controlled and defined animals than are available from commercial breeders or, in some cases, to provide animals that are commercially unavailable.

With the exception of five inbred mouse strains which are maintained by sibling matings, a planned random-bred, monogamous mating system is used. All animals are weighed at weaning and those not within the desired range are discarded. Special disease-control procedures for the breeding colonies include the periodic testing of all breeding pairs for *Pseudomonas aeruginosa*, *Salmonella* and *Citrobacter freundii*-Anl. Those found infected are discarded. All sick, moribund or dead animals are necropsied, and the cause of illness or death is determined, if possible. Discarded breeders from the various colonies and a sampling

weanling discards and 100-day-old stock mice are killed and examined for *P. aeruginosa*, *Salmonella*, *Mycoplasma*, and *Pasteurella pneumotropica*, and for internal and external parasites.

Serological tests for latent viruses, in one strain of mouse (CF#1/Anl) and one strain of rat (SD/Anl), were performed. These tests were conducted by Microbiological Associates, Inc., Washington, D. C. through the courtesy of Dr. Robert Holdenreid of the National Cancer Institute. Discarded breeding animals, 8-12 months of age, were utilized in the serological tests for PVM, Reo 3, GDVII, K, polyoma, Sendai, rat, mouse adeno and mouse hepatitis viruses.

In addition to testing and selection procedures in the conventional colonies, the program includes the initiation of specific-pathogen-free (SPF) colonies. This is accomplished by cesarean sections and foster nursing on pathogen-free animals in plastic film isolators.

PROGRESS REPORT

During the period covered by this report, the rodent-breeding colony has been expanded from 8 strains of mice and rats to 21 strains, five of which are inbred mouse strains for the Janus program. There

are four SPF colonies in production: two CF#1/Anl mouse and two SD rat colonies. Nucleus stocks for three additional SPF mouse colonies have also been initiated.

Of 3,744 breeding-room water bottles cultured for the presence of *Pseudomonas aeruginosa*, only 2 were positive. Of 936 fecal samples cultured for *Salmonella* and *C. freundii*, none was positive. There have been no clinical or pathological findings that would indicate any serious disease problems within any of the breeding colonies. One SPF rat colony did experience a period of extremely low productivity. This was characterized primarily by a sharp increase in neo-

natal deaths. The number of births appeared to be normal but the number weaned was low. This problem disappeared in response to rigorous culling procedures before a definitive cause could be determined.

The results of the serological virus screen tests in the rat colony were negative for all except the Sendai virus for which there was a positive reaction in all 29 rats tested. Similarly, the CF#1/Anl mouse colony was negative to all tests, except for an 8% (4 of 47 tested) incidence of Reo 3 virus.

The rodent-breeding program produced approximately 49,000 mice and 7,000 rats for the Division's use in the past year.

DISEASE CONTROL PROGRAM

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

PURPOSE AND METHODS

The purpose of this program is to provide animals free of all known diseases for the Division's research programs. The several rodent-breeding colonies are continually monitored for the presence of microbial, helminthic, and other metazoan pathogens. Rats and mice purchased from commercial vendors are similarly screened for common bacterial pathogens and parasites. In addition, spontaneous decedents are necropsied to determine the cause of death. Many other control measures are also employed, including the maintenance of a continuous log of the cage and bottle washer operating temperatures, pressure, and causticity; the culturing of sample clean cages and bottles; the operation of a continuous and effective vermin-control program; and the maintenance of accurate records of disease incidence.

PROGRESS REPORT

During the period covered by this report, we observed that the testing of 100-day old stock mice for roundworms is more likely to disclose helminth infected animals than either the testing of 3-4 week old animals or discarded breeders. Consequently, the monitoring of the breeding colonies was modified accordingly.

A weekly analysis of the rodent-colony breeding records (production per pair and weaning percentage)

was also started during the past year and, although it is aimed primarily at increasing the productivity of the colonies, it does have disease control implications. A downward trend in productivity per breeding pair coupled with a decline in percentage weaned would indicate an increase in neonatal deaths and a possible disease problem.

The monitoring of all stock animals and the post-mortem examination of all spontaneous decedents was continued. A new snap-out type necropsy form designed for use with the animal vital statistics computer program was developed. The material recorded on these forms is now being key punched and will be used as a basis for future studies of disease incidence.

A more extensive program of monitoring the health of the experimental animals was also started during the period covered by this report. Each week each investigator is asked to submit a simple report stating whether or not he has observed any signs of illness in his animals. These reports are then visually checked by animal facilities personnel against the animals actually in the facilities to be sure that all have been included. The reports are then compiled, carefully reviewed, and all reported abnormalities are investigated. All animals in the facilities are now included in the disease-control program, and another step in providing an environment for high quality animal research has been achieved.

PATHOGEN STATUS OF COMMERCIALY PRODUCED MICE

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

PURPOSE AND METHODS

Commercially produced laboratory mice frequently are described as "specific-pathogen-free" or simply "pathogen-free," and sometimes as "disease-free." Many commercial breeders advertise their product as "cesarean-derived and barrier-sustained" or as being raised under "rigidly controlled environmental conditions" with the "highest standards of sanitation" including the use of "pasteurized food" and "sterilized bedding." Many advertise that they have a veterinarian on their staff, and in some cases, have laboratory diagnostic control procedures. One should conclude from this array of sophisticated precautions that present-day laboratory mice are carefully quality-controlled and that they are free of most, if not all, disease-producing agents. Except for the survey by Flynn,⁽¹⁾ limited to ectoparasites, and that of Rowe,⁽²⁾ limited to indigenous viruses, there are no published reports of the specific pathogen status of commercially produced laboratory mice.

To see if mice free of common pathogens are now commercially available or if it is still necessary to maintain our own colonies to get healthy animals, sample Lynch-Webster and ICR mice, discarded breeders eight months of age or older, were purchased from 11 colonies maintained by ten nationally-known commercial breeders. On receipt, these *sample* mice were killed and tested for commonly occurring bacterial and parasitic pathogens.

PROGRESS REPORT

P. pneumotropica, an organism associated with conjunctivitis and pneumonia in mice,⁽³⁾ was recovered from the respiratory tract of sample mice in ten of the 11 colonies.

P. aeruginosa, an organism that interferes with radiobiologic research and other studies involving stress⁽⁴⁾ was also cultured from the nasopharynx, lungs or feces of sample mice from ten of the colonies (the negative colony was not the same as the one referred to above).

Only one colony was found to harbor *Mycoplasma* sp., the suspected cause of infectious catarrh of mice,⁽⁵⁾ in the respiratory tract. Conversely, only two colonies were free of respiratory infection with *P. mirabilis*; the clinical significance of this organism is uncertain. Two colonies carried *Achromobacter* sp. in the respiratory tract, and two carried *K. pneumoniae*; both are potential pathogens.

None was found infected with atypical *C. freundii*, an organism that causes an enterocolitis and diarrhea in mice.⁽⁶⁾ Earlier, this organism was recovered from several strains of mice purchased from outside breeders; however, none of the colonies included in the earlier survey was included in this one.

Similarly, no colonies were infected with *Salmonella* sp. This is not surprising in view of the emphasis placed on elimination of salmonellosis from laboratory mice over the years.

The high incidence of uterine infections (18 of 55 mice—32.7%; 9 of the 11 colonies) is certainly disturbing. This infection could be considered insignificant on the basis that the animals tested were discarded breeders and that the kind of animals used for research are usually young nonbreeders were it not for the fact that the incidence in young immature animals has been found to be similar to that of older adult animals from the same colony.^(7, 8) The organism most frequently isolated was *P. pneumotropica* (7 of 55 mice—12.7%; 7 of 11 colonies). This agrees quite closely with the report by Brennan *et al.*⁽³⁾ who found this organism in the uteri of 11.9% of nonproductive female breeders from our colonies and the report by Hoag *et al.*⁽⁹⁾ that 14.6% of nonproductive DBA/2J females at The Jackson Laboratory were similarly infected. The recovery of this organism from the uteri of samples of mice in so many colonies and its concurrent recovery from one of our cesarean-derived, barrier-sustained colonies suggest that intrauterine infection occurs and that the cesarean-section technic may be inadequate to free a colony of this organism.

The majority of the commercial colonies sampled (6 of 11) had mice infected with the roundworm, *A. tetraepthera*. Mice from 2 colonies were also concurrently infected with *S. obvelata*, another oxyurid nematode.

Mice in 6 of the 11 colonies were also infected with ectoparasites. One was infected only with myobiid mites (*Myobia musculi* or *Radfordia affinis*), one only with *Myocoptes musculus* and 4 with both myobiid mites and *Myocoptes*. None was infected with lice (*Polyplax serrata*). In the earlier survey by Flynn,⁽¹⁾ all of 7 colonies sampled were infected with both myobiid mites and *Myocoptes*, and 3 of the 7 were infected with lice. This suggests that some progress has been made by the commercial breeders to rid their stocks of ectoparasites. The 6 colonies infected with ectoparasites are identical with the 6 infected with

endoparasites; the other 5 colonies had no parasites. The presence or absence of parasites seemed to depend on whether or not the colony was initiated by cesarean section.

CONCLUSIONS

A few commercial breeders are producing mice that are relatively free of pathogens, but none that are "pathogen-free." The majority produce animals that carry multiple microbial and metazoan infections, any of which could render them unfit for controlled experimental studies.

Mice free of such infections can be produced. For example, one of our colonies which has been in production for four years is monitored continually by sampling water bottles and feces and by killing and testing sample progeny and discarded breeders; it is free of all common bacterial and parasitic pathogens except for *P. pneumotropica*. Another and newer colony which has been in production for a year and a half is similarly monitored and is completely free of all common pathogens.

MURINE PNEUMONIA

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

PURPOSE AND METHODS

Our continuing studies on the etiology of murine pneumonia led us to attempt the production of the disease using methods proved effective by Nelson.⁽¹⁾

Six Princeton mice were supplied by Dr. J. B. Nelson of the Rockefeller Institute. These mice were infected with a lung suspension of the 42nd *in vivo* passage of a Swiss mouse strain of *Mycoplasma*.

Five days after their arrival at Argonne, the mice developed clinical signs of pneumonia and at necropsy all had lung lesions typical of *Mycoplasma pneumonia*.

TABLE 35. TRANSFER OF MURINE PNEUMONIA
IN CF#1/Anl-SPF MICE

	No. with pneumonia/ No. inoculated	<i>P. pneumotro-</i> <i>pica</i> /ml lung suspension	<i>Mycoplasma</i> / ml lung suspension
Princeton mice	6/6	>100	>100
SPF Transfer ₁	6/6	>100	>100
SPF Transfer ₂	6/6	>100	>100
SPF Transfer ₃	2/8	>10	?
SPF Transfer ₄	0/6	0	0
SPF Transfer ₅	0/6	0	0

It appears that it is still necessary to maintain our own colonies to get healthy mice.

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The lungs were removed, pooled, and a suspension was made. Care was taken to avoid contamination. Cultures of the pooled suspension grew *Pasteurella pneumotropica* and organisms morphologically resembling *Mycoplasma*. Penicillin was added to the suspension at a concentration of 0.1 ml of a 1:10,000 dilution per ml, and 0.03 ml (to kill possible contaminating microorganisms) was inoculated intranasally into CF#1/Anl-SPF mice, a strain free from *P. pneumotropica* infection.

PROGRESS REPORT

The results of five transfers of suspensions of infected lungs to CF#1/Anl-SPF mice are shown in Table 35. From the third transfer, only a few colonies of *P. pneumotropica* were recovered, and it is doubtful whether *Mycoplasma* was isolated. The fourth and fifth transfers were unsuccessful. No disease was produced; no infectious agents were isolated.

These results confirm earlier observations that *P. pneumotropica* is intimately associated with the development of pneumonia in mice.⁽²⁾ Since the strain of Argonne mice used in this study is free from *P. pneumotropica*, it is apparent that the source of the

pneumonia organism was the Princeton mice. It is also evident that this organism is resistant to penicillin at the concentration employed in the inoculum. Since penicillin has been used routinely by Nelson in maintaining murine *Mycoplasma*, the question is whether the primary infectious agent is actually a *Mycoplasma* or whether it is a stable L-form of *P. pneumotropica* produced in response to the addition of penicillin to the suspensions.

Experiments are planned using various concentra-

tions of penicillin to determine if a stable spheroplast of *P. pneumotropica* can be produced. If it can, this L-form will be compared with Nelson's *Mycoplasma*.

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STUDIES OF THE PATHOGENESIS OF THE INTESTINAL LESIONS PRODUCED BY *CITROBACTER FREUNDII*

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

PURPOSE AND METHODS

Since our earlier recognition of *Citrobacter freundii*-An1 as a pathogen for mice,^(1, 2) we have been interested in the disease syndrome produced by this organism, including such related problems as predisposing factors and mode of transmission. In our previous reports^(1, 2) we described the disease syndrome, the etiologic agent, and the lesions in decedents and severely affected animals in both naturally and experimentally infected mice. A logical extension of this work is the study of the pathogenesis of the lesions. In addition to observations of the pathogenesis of gross and microscopic lesions, this study included hematologic examinations and the quantitative and qualitative evaluations of the intestinal flora in these animals.

Twenty-eight CF $\times$ 1/An1 female mice, 4-5 weeks of age, were given 2×10^{13} viable cells of *Citrobacter freundii*-An1 in 0.2 ml of broth by gastric intubation. The mice were maintained in a large (10" x 22" x 6") plastic cage with a stainless steel top. On alternate days, beginning with the second day after infection, two mice were removed from the cage for study. Two uninfected mice were earmarked and added to the cage so that the population of 28 mice was maintained throughout the experiment.

Blood samples were obtained from each animal by anesthetizing it with ether, incising the axillary space and preparing a subcutaneous pouch which was filled by severing the axillary vessels. Total leukocyte, hematocrit, hemoglobin and differential leukocyte counts were made.

After blood samples had been obtained, the abdominal and thoracic cavities were opened by a midline incision from the pubic to mandibular symphyses, and

a necropsy examination of all viscera was conducted using sterile instruments. Observation and the recording of changes in the intestinal tract were given particular critical attention. The changes, classified and recorded in each animal, were vascular congestion of the intestinal mucosa and serosa, external evidence of diarrhea (such as the pasting of the perineum with feces), the quantity of formed feces in the colon, the diameter (dilation) of the colon, and the quality and quantity of mucus in the intestine.

At necropsy, sections of intestine at four levels⁽³⁾ were collected for determination of the intestinal flora.

Tissue samples of duodenum, jejunum, ileum and colon were also collected, fixed in formalin, imbedded in paraffin, both cross and longitudinally sectioned, stained with hematoxylin and eosin, and examined microscopically.

PROGRESS REPORT

The first evidence of diarrhea was observed in a mouse killed on the 8th day. From then until the 18th day, all mice killed showed evidence of moderate diarrhea except for one case in which it was severe. Both mice examined on the 18th day showed severe diarrhea, as did one of the mice examined on the 20th day. The other mouse examined on the 20th day showed only moderate diarrhea. There was no evidence of diarrhea in the mice killed at later times.

At the time that diarrhea was first observed, mean counts of *C. freundii*-An1 jumped from 10^6 per gram of colon to 10^{13} per gram of colon. Counts stayed at this level until the 20th day, when the number of *C. freundii*-An1 dropped to 10^8 in the severely-affected mouse and 10^5 in the moderately-affected mouse. *C. freundii*-An1 was recovered from only one mouse killed

after the 20th day. One mouse killed on the 24th day had 10^4 *C. freundii*-Anl per gram of colon. The colon of this animal was slightly dilated and contained few formed feces, though diarrhea was not evident.

Preliminary examination of the tissue sections revealed changes similar to those described previously.⁽²⁾ Lesions were pronounced in the colon with a slight involvement of the ileum. The first significant changes were noted on the 8th day and consisted of focal infiltration of the submucosa and the lamina propria

FIG. 63.—Total leukocyte counts of mice infected with *Citrobacter freundii*-Anl.

FIG. 64.—Differential leukocyte counts of mice infected with *Citrobacter freundii*-Anl. O, Neutrophile; *, Lymphocyte; +, Monocyte.

with lymphocytes and plasma cells. Small aggregations of neutrophils and small foci of necrosis were also present in the submucosa. From the 8th to the 20th day, the severity of these changes varied widely. The most severe changes were present in one of the animals on day 16. After the 20th day, changes were minimal or absent in all animals. Of some significance was the fact that although abundant mucus was present in the colon of most animals at the time of examination, no evidence of the mucoid-type degeneration of the mucosa of the colon was seen in these animals as contrasted with our previous reports.^(1, 2)

Observations of increased quantity of mucus in the colon, congestion of the mucosa and serosa and dilation of the colon tended to corroborate our previous report that these were primary changes associated with the infection. All of these changes coincided with the onset of diarrhea and the presence of microscopic lesions. Some of these changes, particularly dilation of the colon, seemed to be present at the later stages, after clinical evidence of diarrhea had disappeared and microscopic changes were minimal.

The total leukocyte counts at the time of killing of each mouse are shown in Figure 63 and the differential leukocyte counts may be found in Figure 64. Because of the limited number of animals, these data are inconclusive, but there does appear to be an increase in percentage of neutrophils between day 8 and day 22. This type of inconclusive leukocyte response in clinically sick animals has prompted us to expand our interest in the hematologic response of the CF#1/Anl mouse. While it is true that the numbers of animals in this experiment are small, interpretation of leukocyte responses for diagnostic purposes should be of prognostic and diagnostic value on an individual basis if our understanding of the response and methods of interpretation were adequate.

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INFLUENCE OF INFECTION WITH CITROBACTER FREUNDII ON POSTIRRADIATION SURVIVAL OF MICE

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

PURPOSE AND METHODS

The adverse influence of extraneous, intercurrent or latent disease on experimental animals has been demonstrated many times. Our description^(1, 2) of a diarrheal disease of mice caused by *Citrobacter freundii*-An1 that probably occurs in an inapparent or carrier stage has prompted us to investigate its pathogenicity and its effect on the length of survival of lethally irradiated mice.

Two experiments were conducted during the past year. In the first experiment five groups, each containing 25 12-week-old CF#1/An1 female mice, were used. Four groups were infected by gastric intubation with *C. freundii*-An1, two receiving a high dose (4.6×10^{10} viable cells per mouse) and two receiving a low dose (4×10^5 viable cells per mouse). One of the two groups receiving the high dose and one receiving the low dose and the noninfected group were irradiated with 950 R of x-irradiation at a dose rate of 38.9 R per minute. The number in each group dying on each day after irradiation was recorded. All survivors were killed and necropsied on the 50th day after infection.

In the second experiment, three groups, each containing 40 CF#1/An1 female mice 10–11 weeks old, were used. Two groups were infected by gastric intubation with 2.4×10^{10} viable cells of *C. freundii*-An1.

One of the infected groups plus the noninfected group were irradiated with 950 R of x-irradiation at a dose rate of 39.5 R per minute. The number dying in each group on each day was recorded. All survivors were killed and necropsied on the 20th day after infection.

PROGRESS REPORT

The results (Figures 65 and 66) show that in both experiments mice infected with *C. freundii*-An1 died earlier than those not infected. These data also show that mice in the age range used were resistant to this infection, since the number of deaths was extremely low in the nonirradiated groups in the first experiment and no deaths occurred up to day 20 in the second experiment.

Tables 36 and 37 show the results of culturing the heart blood and colon contents of decedents. In both experiments a high incidence of positive heart cultures was observed suggesting an antemortem septicemia with *C. freundii*-An1. Also, in both experiments survivors in the infected but not irradiated group had lost the organism at the time of examination.

Although the survival time of animals infected with *C. freundii*-An1 is definitely shortened compared to noninfected controls, the difference in survival time is not as great as in animals infected with *Pseudomonas*

FIG. 65.—Mortality record of irradiated mice some of which were infected with *Citrobacter freundii*-An1 before irradiation (Experiment 1).

FIG. 66.—Mortality record of irradiated mice some of which were infected with *Citrobacter freundii*-Anl before irradiation (Experiment 2).

TABLE 36. CULTURE RESULTS (EXPERIMENT 1)

Group	Treatment	<i>Citrobacter freundii</i> recovered/No. decedents cultured	
		Heart	Colon
1	4×10^5 <i>C. freundii</i> -Anl 950 R x-irradiation	1/22*	21/25
2	4.6×10^{10} <i>C. freundii</i> -Anl 950 R x-irradiation	15/24*	25/25
3	950 R x-irradiation	0/25	0/25
4	4×10^5 <i>C. freundii</i> -Anl	(0/4)†	1/1 (0/4)†
5	4.6×10^5 <i>C. freundii</i> -Anl	1/1 (0/4)†	1/1 (0/4)†

* Cannibalism prevented examination of all 25 animals in the group.

† Killed and examined 50 days after infection.

aeruginosa.⁽³⁾ We plan to continue this work, utilizing lower doses of irradiation and we believe that greater differences in survival times between infected and noninfected animals will occur.

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TABLE 37. CULTURE RESULTS (EXPERIMENT 2)

Group	Treatment	<i>Citrobacter freundii</i> recovered/number decedents cultured	
		Heart	Colon
1	950 R x-irradiation	0/40	0/40
2	2.4×10^{10} <i>C. freundii</i> -Anl	—	(1/40)*
3	2.4×10^{10} <i>C. freundii</i> -Anl + 950 R x-irradiation	13/40	34/40

* Killed and examined 20 days after infection.

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STUDIES ON THE PATHOGENICITY OF CITROBACTER FREUNDII

Patricia C. Brennan, Thomas E. Fritz, R. J. Michael Fry,
and Robert J. Flynn

PURPOSE AND METHODS

Several experimental approaches to the problem of the pathogenicity of *Citrobacter freundii*-Anl have been made since our last report. The infection has been studied more extensively in germfree mice and an experiment with suckling mice has been performed to rule out a virus as the etiologic agent of the lesions produced.

In Experiment 1, seven groups of 3-day-old suckling CF#1/Anl mice were inoculated orally with *C. freundii*-Anl, *Clostridium perfringens* and a bacteria-free filtrate of infected colons in all possible combinations (Table 38). The inocula were prepared according to the method of Brennan, *et al.*⁽¹⁾ An eighth group was given 0.03 ml of the filtrate intraperitoneally and a ninth group of HA/ICR 6-week-old germfree mice was given 0.2 ml of the filtrate by gastric intubation.

In Experiment 2, three groups of germfree HA/ICR mice were inoculated with 1) 2.6×10^{11} *C. freundii*-Anl, 2) 6×10^{11} *C. perfringens*, and 3) a combination of these two organisms at the same dose. Groups of conventional animals were similarly treated.

In Experiment 3, two groups of HA/ICR germfree mice were inoculated with 1) 2.4×10^{10} *C. freundii*-Anl and 2) 2.4×10^{10} *C. freundii*-Anl plus 2.6×10^9 *C. perfringens*. Because of the results of Experiment 2, more elaborate conventional control groups were used that consisted of the following treatments:

1. HA/ICR *C. freundii*-Anl by gastric intubation
2. HA/ICR *C. freundii*-Anl + *C. perfringens* by gastric intubation
3. HA/ICR *C. freundii*-Anl in the drinking water for 24 hours
4. HA/ICR *C. freundii*-Anl + *C. perfringens* in the drinking water for 24 hours
5. CF#1/Anl *C. freundii*-Anl by gastric intubation
6. CF#1/Anl *C. freundii*-Anl + *C. perfringens* by gastric intubation

PROGRESS REPORT

Table 38 shows the results of the first experiment. Diarrhea did not develop until at least 7 days after inoculation. Those groups which received the filtrate with *C. freundii*-Anl did not exhibit clinical signs of disease until the 13th day after infection. The filtrate alone did not affect any group. Most deaths in *C. freundii*-Anl-inoculated animals occurred between the 20th and 25th day after inoculation. This is well be-

yond the time after inoculation that mice die from the lethal intestinal virus reported by Kraft.⁽²⁾

In the second experiment, none of the germfree mice developed diarrhea or exhibited lesions at necropsy. This is consistent with our previous finding that *C. freundii*-Anl, as a monocontaminant, is nonpatho-

TABLE 38. *Citrobacter freundii*-Anl INFECTION IN SUCKLING MICE

Experimental group	Day of onset of illness	Number showing illness/number inoculated	Number from which <i>C. freundii</i> -Anl recovered/number inoculated	Number from which <i>C. perfringens</i> recovered/number inoculated
Filtrate	—	0/6	0/6	0/6
<i>C. freundii</i> -Anl	9	9/9	9/9	8/9
<i>C. perfringens</i>	—	0/5	0/5	5/5
<i>C. freundii</i> -Anl + <i>C. perfringens</i>	7	9/9	9/9	9/9
Filtrate + <i>C. freundii</i> -Anl	13	5/5	5/5	0/5
Filtrate + <i>C. perfringens</i>	—	0/5	0/5	5/5
Filtrate + <i>C. freundii</i> -Anl + <i>C. perfringens</i>	13	9/9	9/9	9/9
Filtrate IP	—	0/7	0/7	5/7
Filtrate in germfree mice	—	0/10	0/10	0/10

TABLE 39. *Citrobacter freundii*-Anl INFECTION IN CONVENTIONAL HA/ICR AND CF#1/Anl MICE

Experimental group	HA/ICR		CF #1/Anl	
	Number from which <i>C. freundii</i> -Anl recovered/number cultured	Number with lesions/number examined	Number from which <i>C. freundii</i> -Anl recovered/number cultured	Number with lesions/number examined
<i>C. freundii</i> -Anl (stomach tube)	8/9	1/9	5/5	5/5
<i>C. freundii</i> -Anl + <i>C. perfringens</i> (stomach tube)	5/9	0/9	6/6	6/6
<i>C. freundii</i> -Anl (water bottle)	6/6	2/6	—	—
<i>C. freundii</i> -Anl + <i>C. perfringens</i> (water bottle)	5/6	3/6	—	—

genic.⁽³⁾ Mice contaminated with *C. perfringens* in addition to *C. freundii*-Anl failed to develop the disease; the reason is not clear. Apparently *C. perfringens* alone does not alter intraluminal conditions sufficiently for *C. freundii*-Anl to express its pathogenicity. That conventional mice did not develop the disease was unexpected, since mice of this strain became ill in an earlier experiment. *C. freundii*-Anl was neither recovered from the feces of infected HA/ICR conventional mice nor was it recovered at necropsy.

In the third experiment, the conventional HA/ICR mice developed catarrhal enterocolitis, but a large inoculum in the water bottles was usually required to produce the disease, and only 6 out of 30 mice had lesions (Table 39). Although *C. freundii*-Anl was recovered in large numbers from the animals with lesions, it was also recovered from the unaffected animals. In the unaffected animals, it was present in much fewer numbers, and other coliforms were also recovered. All CF#1/Anl mice of the same age, weight and sex developed clinical signs of disease, and all had typical

lesions. No reason is known for the observed strain differences in susceptibility to this disease between CF#1/Anl and HA/ICR mice. Besides the genetic differences between the two strains, differences in the normal intestinal flora may exist. Also, the *Escherichia coli* carried by HA/ICR mice may be a colicin-producing strain and may make the implantation of *C. freundii*-Anl difficult. Studies are now in progress to compare the normal flora in the two strains of mice. We are also studying this infection in germfree CF#1 mice.

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THE INTESTINAL MICROFLORA OF THE CF#1/ANL MOUSE

Patricia C. Brennan, Judith A. Giolitto,* Thomas E. Fritz,
and Robert J. Flynn

PURPOSE AND METHODS

Alteration of the intestinal microflora is thought to produce changes in an animal's response to external stimuli. Altered response to endotoxins, infections, and radiation damage have been attributed to a change in the intestinal flora of mice.⁽¹⁻⁵⁾ Unfortunately, most of the published information is based only on fecal flora. In those instances where attempts have been made to examine the entire intestine they involved only a few animals,⁽⁶⁾ or the data are presented in a fashion that makes comparison^(7, 8) with results from other laboratories impossible. Therefore, the purpose of this investigation has been 1) to develop precise and practical methods for the qualitative and quantitative determination of the intestinal flora; 2) to determine whether significant differences in population size and in the kinds of bacteria exist in various anatomical regions of intestine; and 3) to establish standard values for the different regions which can be used as a base for comparing the effect of experimental treatment on the flora.

CF#1/Anl male and female mice 10-12 weeks of

age were used. They were fed a commercial pelleted mouse diet, and water was given ad libitum. The mice were housed 15 per cage prior to study.

When mice were killed, the entire small intestine from each mouse was removed aseptically and placed in a sterile petri dish. Three segments of small intestine were taken, one in the duodenum approximately 1 cm from the pylorus, one in the ileum approximately 1 cm from the ileocecal junction and a third in the jejunum midway between the other two segments. Each segment weighed approximately 0.2 g. A fourth segment was taken from the middle of the colon. The segments were opened and both the contents and the gut itself were placed in 0.4 ml sterile Reinforced Clostridia Medium (RCM) broth. Ten-fold serial dilutions were made in 0.9 ml RCM broth, 0.02 ml of each dilution was plated on five selective media according to the method of Miles and Misra.⁽⁹⁾ Final dilutions plated were from 10^{-2} - 10^{-12} . Plates were incubated under conditions most favorable to the group of microorganisms sought. The counts were made from the dilutions as follows:

- 1) The dilutions that produce discrete colonies were determined.
- 2) Counts were made from the selective media.

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- 3) The mean of counts on each medium was calculated and reported as *the count* for the individual organism (micrococci, coliforms, enterococci, etc.) recovered from the animal from which the culture was obtained.

Final assignment of an isolate on a particular selective medium to a genus or family was based on its possession of characteristic taxonomic features.

A subjective evaluation of the quantity of material in the gut was made. A full segment was assigned a value of 4+; an empty one was considered 0.

PROGRESS REPORT

Table 40 shows the quantity of material in the segment of intestine compared with the total number of organisms it contained. The total counts are, in general, a reflection of the number of lactobacilli and Bacteroidaceae, or both. In the colon, terminal ileum and jejunum, the quantity of material in the gut had an effect on the number of organisms recovered. Higher counts were observed in filled segments of the colon

TABLE 40. COMPARISON OF THE QUANTITY OF INTESTINAL CONTENT TO THE NUMBER OF MICROORGANISMS PER SECTION OF INTESTINE IN THIRTY CF #1/Anl MICE

Log of viable organisms	Fullness of duodenum					Fullness of jejunum					Fullness of ileum					Fullness of colon				
	0	1	2	3	4+	0	1	2	3	4+	0	1	2	3	4+	0	1	2	3	4+
	Number of animals					Number of animals					Number of animals					Number of animals				
12													1	4				2		9
11								1	2								1	1		
10						1		1	1					1				1	1	
9								1	2					1	3			1		
8	2	1				1	1	2			2	1	2			1				1
7	3	2						3	2				2	3				4	3	
6	4	3				2	2		1				3	2				1		1
5	3	2	1			1	2				1						1			
4	5					2	1				1	1								
3	3		1			1					1									
2											1						1			
<2																	1			

and terminal ileum than in segments that were nearly empty. This relationship was not as evident or reliable in the jejunum and was not observed in the duodenum.

Table 41 shows the mean count for five groups of microorganisms per section of intestinal segment.

The extreme variability in the numbers of microorganisms among individual animals is apparent from the high standard error obtained; as much as 69% of the mean for the Bacteroidaceae. This is probably a reflection of the fastidious nature of this group of organisms as well as the variability among animals. It is also quite possible that the variation encountered accounts for the lack of published quantitative data.

Although statistical evaluation of these results is not complete, some general statements seem justified. At the 5% level of significance, no difference in the number of micrococci at any gut level is evident. Significantly higher counts of enterococci and Bacteroidaceae were obtained in the jejunum, ileum and colon than in the duodenum. The number of enterococci recovered was significantly greater in the colon than in the jejunum but no difference in number was noted between the jejunum and ileum or the ileum and colon. The Bacteroidaceae were significantly more numerous in the colon than in the jejunum and ileum but not in the ileum than the jejunum. These results indicate that the Bacteroidaceae and enterococci preferentially inhabit the terminal portions of the intestine. The Bacteroidaceae are by far the most numerous organisms in the colon.

Low numbers of coliforms were recovered in all segments. No difference was noted in numbers between adjacent sections of small intestine but differences do exist among the duodenum, ileum and colon and between jejunum and colon. Interestingly a significant increase in coliforms was found from the ileum to the colon. This is probably a reflection of the stasis which obtains in the colon.

The lactobacilli were most numerous in the colon, but high numbers exist throughout the gut. The only significant difference in counts was between the duodenum and the other three segments.

TABLE 41. BACTERIAL POPULATION IN ANATOMICAL REGIONS OF THE MOUSE INTESTINE

Organism	Duodenum	Jejunum	Ileum	Colon
Micrococci	$1.5 \times 10^2 \pm 15^*$	$1.7 \times 10^2 \pm 11$	$2.8 \times 10^2 \pm 23$	$7.2 \times 10^2 \pm 21$
Enterococci	$4.8 \times 10^3 \pm 34$	$4.9 \times 10^4 \pm 46$	$1.4 \times 10^5 \pm 48$	$4.6 \times 10^6 \pm 55$
Coliforms	$1.9 \times 10^2 \pm 20$	$7.0 \times 10^2 \pm 20$	$3.4 \times 10^3 \pm 33$	$8.0 \times 10^4 \pm 54$
Lactobacilli	$1.0 \times 10^6 \pm 30$	$2.5 \times 10^7 \pm 45$	$8.4 \times 10^7 \pm 55$	$5.3 \times 10^8 \pm 67$
Bacteroidaceae	$4.2 \times 10^3 \pm 20$	$1.5 \times 10^5 \pm 36$	$2.6 \times 10^5 \pm 50$	$8.7 \times 10^8 \pm 69$

* Results expressed as the mean population per section of intestine in 30 CF #1/Anl mice $\pm$ the percent standard error.

Bacteria other than these five major groups were also isolated in this study. Clostridia were present in 80% of the animals examined and were present in low numbers in all regions of the gut. Anaerobic streptococci were recovered from 20% of the animals; however, it is expected that most animals harbor them and their low incidence is due to the media employed. Only one animal harbored *Proteus* at a level of 10^2 /g or better. This is interesting because these organisms are usually thought to be a major constituent of the intestinal flora and are common in tissues from animals exhibiting any degree of postmortem decomposition at the time of collection.

CONCLUSION

Despite the extreme variability among mice in the numbers of microorganisms recovered from the several segments of the intestine, it is possible to compare the specific regions. It is also possible to show that differences in numbers exist in different regions and that some microorganisms preferentially inhabit a particular portion of the gut. The values obtained provide a basis for the study of experimental effects on the flora of the CF#1/Anl mouse.

THE EFFECT OF NEOMYCIN AND X-IRRADIATION ON THE INTESTINAL MICROFLORA OF THE CF#1/ANL MOUSE

Patricia C. Brennan, Judith A. Giolitto,* Thomas E. Fritz, and Robert J. Flynn

PURPOSE AND METHODS

Using the methods described in the preceding paper we have studied the effect of experimental treatment on the intestinal microflora.

Three groups of CF#1/Anl mice were used. In Experiment 1, neomycin at a concentration of 2 mg/ml was added to the drinking water. After 3 days, the mice were killed and examined. In Experiment 2, the mice were examined 3 days after irradiation; and in Experiment 3, they were examined 10 days after irradiation.

PROGRESS REPORT

Tables 42-44 show the results of the three experiments. Neomycin reduced microbial populations at all levels of the intestine. Enterococci were not recovered from the ileum in any animal and were present in small numbers in other sections. Coliforms were re-

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covered only in the duodenum, although in untreated animals they are generally confined to the colon. Lactobacilli were significantly more numerous in the upper intestine than in the terminal portions of the intestine, a reversal of the conditions that exist in control animals. Bacteroidaceae were greatly reduced in numbers but were still present in all sections; this is believed to be the result of the resistance of this group of organisms to the antimicrobial action of neomycin. It has been claimed that the colon is "sterilized" by the action of neomycin. We did not find this in mice, although we did observe that populations were reduced in the colon in comparison with anterior sections of gut.

We can say little about the effect of x-irradiation until the statistical analysis is complete. However, there is a reduction in the number of coliforms in the colon at 3 days after irradiation and this reduction even more marked at 10 days after irradiation.

Future work will include the effect of other anti-

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TABLE 42. BACTERIAL POPULATION IN ANATOMICAL REGIONS OF THE MOUSE INTESTINE AFTER THREE-DAY TREATMENT WITH NEOMYCIN*

Organism	Duodenum	Jejunum	Ileum	Colon
Micrococci	Not recovered	$9.6 \times 10^1 \pm 6$	$7.9 \times 10^1 \pm 5$	$7.8 \times 10^1 \pm 5$
Enterococci	$9.1 \times 10^1 \pm 12^\dagger$	$1.2 \times 10^2 \pm 20$	Not recovered	$7.2 \times 10^1 \pm 6$
Coliforms	$6.3 \times 10^1 \pm 2$	Not recovered	Not recovered	Not recovered
Lactobacilli	$1.4 \times 10^4 \pm 29$	$3.0 \times 10^4 \pm 43$	$8.0 \times 10^2 \pm 31$	$1.8 \times 10^2 \pm 17$
Bacteroidaceae	$1.8 \times 10^2 \pm 21$	$5.2 \times 10^2 \pm 29$	$2.5 \times 10^3 \pm 47$	$7.0 \times 10^5 \pm 84$

* Mice received neomycin sulfate in their drinking water at a concentration of 2 mg/ml.

† Results are expressed as the mean population per section of intestine in 28 CF #1/Anl mice $\pm$ the percent standard error.

TABLE 43. BACTERIAL POPULATION IN ANATOMICAL REGIONS OF MOUSE INTESTINE FOUR DAYS AFTER 700 R X-IRRADIATION

Organism	Duodenum	Jejunum	Ileum	Colon
Micrococci	$1.1 \times 10^2 \pm 16^*$	$1.3 \times 10^2 \pm 18$	$3.6 \times 10^2 \pm 18$	$1.8 \times 10^3 \pm 30$
Enterococci	$1.1 \times 10^4 \pm 30$	$3.5 \times 10^4 \pm 32$	$4.0 \times 10^5 \pm 52$	$5.0 \times 10^6 \pm 48$
Coliforms	$1.1 \times 10^2 \pm 13$	$1.8 \times 10^2 \pm 20$	$1.5 \times 10^3 \pm 34$	$3.9 \times 10^3 \pm 34$
Lactobacilli	$4.6 \times 10^5 \pm 29$	$3.1 \times 10^6 \pm 32$	$5.2 \times 10^7 \pm 55$	$1.9 \times 10^9 \pm 49$
Bacteroidaceae	$2.5 \times 10^4 \pm 25$	$1.7 \times 10^5 \pm 27$	$6.7 \times 10^5 \pm 43$	$3.4 \times 10^9 \pm 49$

* Results are expressed as the mean population per section of intestine in 30 CF #1/Anl mice $\pm$ the percent standard error.

TABLE 44. BACTERIAL POPULATION IN ANATOMICAL REGIONS OF MOUSE INTESTINE TEN DAYS AFTER 700 R X-IRRADIATION

Organism	Duodenum	Jejunum	Ileum	Colon
Micrococci	$7.0 \times 10^1 \pm 4^*$	$1.9 \times 10^2 \pm 28$	$3.9 \times 10^2 \pm 19$	$1.9 \times 10^3 \pm 20$
Enterococci	$6.2 \times 10^3 \pm 35$	$2.3 \times 10^5 \pm 58$	$8.8 \times 10^5 \pm 53$	$3.9 \times 10^7 \pm 65$
Coliforms	$7.8 \times 10^1 \pm 5$	$4.7 \times 10^2 \pm 36$	$1.4 \times 10^3 \pm 38$	$3.5 \times 10^3 \pm 41$
Lactobacilli	$2.2 \times 10^6 \pm 37$	$5.8 \times 10^7 \pm 55$	$1.2 \times 10^9 \pm 65$	$4.4 \times 10^{10} \pm 58$
Bacteroidaceae	$6.3 \times 10^3 \pm 40$	$1.1 \times 10^6 \pm 65$	$6.2 \times 10^6 \pm 84$	$7.6 \times 10^9 \pm 75$

* Results are expressed as the mean population per section of intestine in 30 CF #1/Anl mice $\pm$ the percent standard error.

biotics on the intestinal flora of the CF#1/Anl mouse. The effect of different levels of irradiation and exami-

nations at varying times after irradiation on this mouse will also be studied.

FLUORESCENT ANTIBODY TECHNIQUE AS A SCREENING TOOL

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

PURPOSE AND METHODS

The procedures at present employed to screen the Division's rodent colonies for *Citrobacter freundii*-Anl and *Mycoplasma* require from 3 to 7 days and limit the examination to only a few (5-10) animals per week. It is an advantage to have a method that gives results in 24 hours and that permits the screening of 10-25% of the colony per week. Therefore, we devised a method utilizing the fluorescent antibody (FA) technique to solve our problem.

Antiserum was prepared against *C. freundii*-Anl using the method of Edwards and Ewing⁽¹⁾ described for

Shigella. The indirect antibody test of Cherry, Goldman and Carski⁽²⁾ was employed in preliminary studies.

Antisera against six strains of *Mycoplasma* were prepared according to the method of Clark *et al.*⁽³⁾ Preliminary studies have used their method for the indirect test.

PROGRESS REPORT

Results with *C. freundii*-Anl antiserum indicate that cross reactions with other *Enterobacteriaceae* are not adequate to negate the usefulness of the procedure.

The atypical strain of *C. freundii* cross-reacts with *Salmonella typhimurium* and *S. anatum*. Since *Salmonella* have been isolated from the Division's mice only once in two years, this cross reaction is not important. The antiserum does not cross react appreciably with *Escherichia coli*, *Aerobacter aerogenes* or *Proteus*, the common intestinal enterobacteria.

It would seem, therefore, that *C. freundii*-AnI could be detected in fecal smears. In a preliminary investigation, mice from a commercial supplier were examined by the FA technique in addition to the conventional method. Mice from this supplier had previously been shown to harbor *C. freundii*-AnI in the intestinal tract. Examination of fecal smears from this source stained with fluorescent antibody showed numerous fluorescent microorganisms. None was observed in smears from mice raised in the Division and free of *C. freundii*-AnI. Culture results confirmed the presence of *C. freundii*-

AnI in the positive smears. Future studies will include the examination of a large number of infected and noninfected mice to determine the minimum number of *C. freundii*-AnI which will give positive results.

Results with *Mycoplasma* antisera have been discouraging. It has not been possible to repeat the work of Clark and his coworkers because of cross and non-specific reactions. It is hoped that these technical difficulties can be overcome by the direct FA technique.

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ANIMAL VITAL STATISTICS COMPUTER PROGRAM

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PURPOSE AND METHODS

The animal vital statistics program, utilizing computer systems for data recording and processing, is directed toward a method of more efficiently kept rodent breeding records, a critical analysis of the breeding efficiency of the colonies, the collection of data on the incidence of diseases, and a continuous check on the health status of animals intended for experimental use. It should be helpful in the detection of trends and identification of leads for the investigation of subtle or insidious causes of death or morbidity in laboratory animals.

PROGRESS REPORT

During the period covered by this report, several additions and refinements have been made to the program. It now includes a method for the weekly listing and identification of all breeding rodents in the production colonies. Based on this listing, two methods for evaluating the breeding efficiency of pairs of animals and for culling the nonproductive pairs have been added. The ability to list all animals is also valuable for checking the accuracy of the stored data. Included are the birth date of the female, her age in days, the

date of her last litter, and the number of days elapsed since her last litter.

By machine sorting of the output prepared from this listing, we are able to cull those animals that have not produced a litter over a given time interval or by any selected age. All breeding pairs that do not produce their first litter by 150 days of age or subsequent litters at 45-day intervals are culled.

As part of this program, a necropsy record keeping system has been devised and put into use during the last year. This new system utilizes a multipart necropsy form designed for punch-card recording and sorting of the data collected. The standard "Nomenclature of Veterinary Diseases and Operations," compiled and published by the National Cancer Institute, is used for the numerical coding of all diagnoses. The new system is intended to allow retrieval of diagnoses and pertinent laboratory data not now easily retrievable because of the large number of examinations conducted. Valuable and unique information on the incidence of diseases will be provided. These statistics will be useful, not only in the laboratory animal disease research program, but also in evaluating the health status of animals intended for research.

TOXICITY AND METABOLISM OF RADIONUCLIDES

TOXICITY AND METABOLISM OF RADIONUCLIDES: PROGRAM OBJECTIVES AND INTRODUCTION

William P. Norris

The objective of this program is the systematic study, primarily using the beagle dog, of the effects of selected fission products administered singly or as well-defined, simple mixtures. Radionuclides selected for study were chosen as being representative of one of the following types: 1) "biologically soluble" radioisotopes which will irradiate an entire animal more or less uniformly; 2) "biologically insoluble" radioisotopes which are preferentially taken up by the phagocytic cells of the reticuloendothelial system and, to some considerable extent, by bone; 3) "bone seekers" which are almost entirely restricted to the skeleton; 4) iodine, which by virtue of its chemistry is preferentially deposited in the thyroid gland.

Representatives of Classes 1 and 2— Cs^{137} and Ce^{144} , respectively—have been given intravenously in single doses to pure-bred beagle dogs. The results have served to define the picture of acute lethality of these radionuclides in dogs. These isotopes have also been administered in doses less than those found acutely lethal for the demonstration of their late effects.

The study of radiation damage produced by administered fission products is fundamentally one of the effects of protracted irradiation. However, the radiation dose is usually nonuniformly distributed throughout the body and decrementing with time, depending on the physical and biochemical properties of the radionuclide in question. Since Class 1 radionuclides deliver radiation dosages in a reasonably uniform manner to the entire body, it is of interest to compare the effects produced by injected Cs^{137} to those produced by whole-body irradiation using an external γ -ray source.

A large share of this year's effort has been devoted to the evaluation of the response of the dog to single

or protracted doses of Co^{60} γ rays delivered to the whole body. This work was accompanied by the necessary effort using microdosimetric techniques required to begin the comparisons mentioned earlier.

During the past two months, a limited experiment was initiated to define doses of I^{131} which produce visible pathology in the dog, ranging from minimal damage to ablation of the thyroid gland. No further report of this work is included in the following text, since results are not yet available. It is clear that a study of the effects of irradiation from sources composed of mixtures taken from Classes 1-4 and external whole-body irradiation will do much to elucidate the hazards of mixed fission products. Such an experimental study will begin as soon as sufficient information is compiled using members of these classes singly.

It is important to view these animals in sufficient detail to obtain a comprehensive picture of alterations in their biochemistry, physiology, clinical status, radioactive content, pathological changes, and radiation dosimetry. To the extent permitted by the present staff, these observations are being made, and the results will be included in the following reports.

Experience to date has demonstrated the importance of uniform, genetically defined, dogs in this type of experimental work. To this end, this beagle colony has remained closed since it was established, and two separate lines of inbred dogs have been maintained within the colony. Several genetically transmitted anomalies, not confined to the inbred lines, have been recognized and studied in varying detail. The first summarization in this area is included under the Genetics section of this report in a paper by Rehfeld and Grahn on the incidence of atape-tal beagles in a large colony.

CESIUM-137—TOXICITY AND INDUCED PATHOLOGY IN DOGS OF DIFFERENT AGES

Louise S. Lombard,* Robert Zaldwar, William P. Norris, Norbert D. Kretz, and Calvin M. Poole

PURPOSE AND METHODS

A total of 73 beagle dogs was given Cs¹³⁷ intravenously in doses near that found to be lethal within 30 days after injection. The pertinent information concerning doses of injected Cs¹³⁷ and survival is contained in Table 45. These animals fall into three age groups at time of treatment: 5 months, 13 months, and 4–5 years.

During the last year, the surviving animals have been followed clinically. The study of induced pathology in those which failed to survive the early period after treatment is essentially complete.

PROGRESS REPORT

In the annual report of this Division for 1964,⁽¹⁾ it was shown that the differences in Cs¹³⁷ toxicity, as a function of age at injection, is primarily due to the slower excretion of cesium by older animals. Hence, older animals appear to be more susceptible. However, the average computed radiation dose to death at around 30 days was the same for all age groups. Animals died in this period from an integrated radiation dose computed to be 900 rads to the whole body. Those which survived accumulated well in excess of 1000 rads. A more detailed examination of dosimetry in these animals is in progress.

During the course of making clinical examinations of the dog colony, it became evident that the testicles of the surviving, Cs¹³⁷-injected males were much smaller than those of untreated males. To evaluate this condition, external length and width measurements were made of the testicles of all 24 surviving experimental dogs and compared to similar values from untreated animals. These measurements revealed that the testicular volume of the average, Cs¹³⁷-treated male was slightly less than half that of the control dogs. In order to characterize the change further, semen samples were obtained from 15 of the Cs¹³⁷ dogs and from 4 control dogs by manual ejaculation. The males which were ejaculated all exhibited normal libido. Although samples were unobtainable from 9 experimental males, this did not appear to be due to lack of sex drive, but rather to temperament or behavioral problems such as shyness or aggressiveness. None of the samples from the Cs¹³⁷ dogs, regardless of the dose level, contained any sperm cells. The samples were clear to slightly milky in color

and quite watery in appearance. The semen from the controls was a milky, more viscous fluid that contained highly motile sperm cells. The sperm counts on these samples ranged from 87,200 to 430,000 per mm³.

These specific, testicular observations were made in October and November of 1964. Current visual observations indicate that there has been no marked change in the testicular atrophy. With this exception, all the survivors appear to be in good health at this time. Elapsed time since treatment ranges from 3 to 4.5 years.

At necropsy of those dogs which failed to survive, hemorrhages were found in all organs with few exceptions. Hemorrhagic extravasations (lesions measuring 125 mm in diameter and over) and effusions appeared in the dermis and subcutis of the head and neck, intestinal mucosa, and body cavities. Hemothorax, hemopericardium, and hemoperitoneum were common findings in dogs of all age groups. The testes exhibited unusually large hemorrhagic areas.

The histopathologic study was based on numerous tissue samples from almost all the organs of the body. Only the lesions in the two more important groups (13-month and 46 to 68-month) will be included here.

Thirteen-month Age Group

In the musculoskeletal system, areas of atrophy, degeneration, and necrosis were noted.

The respiratory system exhibited ulcerated areas in the mucosa of the nasal sinuses. Exudate in the alveoli of the lungs was present and consisted of erythrocytes, macrophages with lipid and hemosiderin, plasma cells, fibrin and bacteria. Lipid-bearing macrophages were seen within the walls of the alveoli and bronchi.

In the circulatory system, several myocardial changes were noted, including vascular emboli, infarcts and myopathy. Emboli were found within the venules of the myocardium as well as in all or almost all the viscera. Round to oval intravascular vacuolated bodies, most of which were PAS positive and were undigested by diastase, were found. Such bodies measured from 10 to 100 microns.⁽²⁾ Gram-positive and gram-negative bacteria were observed intravascularly in the tissues of several dogs.

The digestive system showed severe changes. This consisted mainly of ulcers in the mucosa of the tongue, esophagus, stomach, and intestines as well as loss of lymphoid tissue in the patches of Peyer. The acces-

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sory digestive organs also exhibited abnormalities. In the liver, there was congestion, bile, and ceroid pigment in the cytoplasm of hepatocytes, lipid, mucopolysaccharides, iron, and erythrocytes within the

Kupffer cells, necrotic areas with cysts and bacteria, as well as necrosis in the mucosa of the gall bladder. Fatty change in hepatic cells was also observed.

The main lesions of the hemopoietic system con-

TABLE 45. SURVIVAL OF BEAGLES FOLLOWING A SINGLE INTRAVENOUS DOSE OF Cs¹³⁷

Dog No.	Sex	Date injected	Age at injection, days	Amount		Days to death	Survival* at 10-1-65	Cause of death
				mCi	mCi/kg			
5-Month-old dogs								
470	M	10-23-62	151	25.22	3.85	27	1074	Bone marrow aplasia
308	M	4-17-62	151	32.27	3.79			
473	F	10-23-62	151	21.22	3.79		1074	
492	F	10-31-62	148	22.10	3.76		1066	
471	M	10-23-62	151	21.91	3.76		1074	
487	M	10-31-62	149	25.79	3.70	39		Bone marrow aplasia
401	F	4-17-62	151	29.40	3.68	27		Bone marrow aplasia
397	M	4-17-62	151	35.29	3.67		1263	
404	F	4-17-62	142	30.52	3.65		1263	
491	F	10-31-62	148	20.09	3.65		1066	
472	F	10-23-62	151	17.19	3.65		1074	
485	M	10-31-62	149	27.50	3.64		1066	
474	F	10-23-62	151	23.80	3.60		1074	
486	M	10-31-62	149	24.57	3.59		1066	
399	M	4-17-62	151	30.26	3.24		1263	
13-Month-old dogs								
417	M	2-5-63	388	45.68	4.31	27		Bone marrow aplasia
418	M	2-5-63	388	44.31	4.28	26		Bone marrow aplasia
416	F	2-5-63	388	44.16	4.25	24		Bone marrow aplasia
419	M	2-5-63	388	36.61	4.14	27		Bone marrow aplasia
241	F	1-18-62	417	26.48	3.69	31		Bone marrow aplasia
237	F	1-18-62	417	31.91	3.59	28		Bone marrow aplasia
438	M	5-1-63	391	37.83	3.54		884	
437	M	5-1-63	391	33.57	3.46		884	
392	F	12-5-62	395	26.77	3.45		1031	
393	F	12-5-62	395	23.85	3.41		1031	
435	M	5-1-63	391	35.01	3.40		884	
436	M	5-1-63	391	35.34	3.40		884	
239	M	1-18-62	417	34.44	3.36		1352	
391	F	12-5-62	395	28.03	3.34		1031	
243	M	1-18-62	417	40.22	3.33	35		Bone marrow aplasia
390	F	12-5-62	395	29.27	3.31		1031	
313	F	11-6-62	561	27.73	3.18	7		Euthanized for tissue study
366	F	11-6-62	399	24.26	3.17		1060	
316	F	11-6-62	561	28.34	3.14	14		Euthanized for tissue study
369	M	11-6-62	399	24.52	3.10	28		Bone marrow aplasia
367	M	11-6-62	399	25.91	3.09	36		Bone marrow aplasia
450	M	5-21-63	427	25.00	2.84		904	
428	F	5-21-63	415	21.49	2.72		904	
441	F	5-21-63	413	27.31	2.70	52		Bone marrow aplasia
451	M	5-21-63	427	29.05	2.70		904	

TABLE 45. *Continued*

Dog No.	Sex	Date injected	Age at injection, days	Amount		Days to death	Survival* at 10-1-65	Cause of death
				mCi	mCi/kg			
13-Month-old dogs—Continued								
452	M	5-21-63	427	27.91	2.68	38	904	Euthanized for tissue study (Tumor of left ovary removed)
430	F	5-21-63	415	26.55	2.65		904	
449	M	5-21-63	418	33.20	2.65			
246	F	9-19-61	398	22.52	2.43		1473	
258	F	9-19-61	404	24.66	2.32		1473	
256	F	9-19-61	404	18.50	2.27	0 2	1473	Accidental death Accidental death
257	F	9-19-61	404	20.20	2.23		1473	
247	F	9-19-61	398	21.86	2.16		1473	
55	M	4-11-61	397	17.17	2.11		1634	
53	M	4-11-61	415	22.62	2.11			
52	M	4-11-61	415	17.56	2.11			
54	M	4-11-61	397	17.05	1.98		1634	
81	M	4-11-61	397	15.52	1.85		1634	
444	M	5-21-63	413	18.52	1.74		904	
443	M	5-21-63	413	18.09	1.73		904	
439	M	5-21-63	415	17.59	1.70		904	
453	F	5-21-63	435	18.42	1.66		904	
454	M	5-21-63	435	16.33	1.65		904	
Older Dogs								
46	F	9-25-62	1387	36.65	3.74	21		Bone marrow aplasia
69	M	9-25-62	1662	42.45	3.71	24		Bone marrow aplasia
72	M	9-25-62	1662	34.60	3.70	22		Bone marrow aplasia
71	M	9-25-62	1662	30.49	3.70	22		Bone marrow aplasia
48	F	9-25-62	1509	33.60	3.64	20		Bone marrow aplasia
65	M	11-20-62	2060	24.43	3.40	28		Bone marrow aplasia
60	F	11-20-62	2066	23.11	3.39	24		Bone marrow aplasia
63	F	11-20-62	2060	25.23	3.32	26		Bone marrow aplasia
64	M	11-20-62	2060	19.75	3.29	29		Bone marrow aplasia
80	M	11-20-62	2060	28.16	3.27	24		Bone marrow aplasia
107	M	2-16-61	ca. 1023	26.32	1.99	64		Bone marrow aplasia
121	M	2-16-61	ca. 1023	28.09	2.04	27		Bone marrow aplasia
124	M	{ 2-16-61 10-2-61	ca. 870	{ 20.40 16.15	{ 1.58 1.04		1688	
114	M	{ 10-31-60 8-14-61 4-10-62	ca. 731	{ 7.80 10.76 11.63	{ 0.93 1.01 1.13		1796	
128	M	{ 10-31-60 8-14-61 4-10-62	ca. 762	{ 11.75 11.89 12.83	{ 1.08 0.99 1.01		1796	

* Living at the time of reporting except as otherwise noted.

sisted of aplasia of the bone marrow in those dogs dying within approximately 30 days postinjection and hypoplasia in the one dying at 52 days postinjection.

The endocrine glands had, as principal changes, atrophy of the adrenal cortex as well as loss of lipid

and necrotic foci in the adrenals. Necrotic cells within the islets of Langerhans were also seen.

In the urogenital system, severe changes were noted, including foci of necrosis within the glomerular tuft as well as necrosis of segments of the proximal

convoluted tubules. The testes of several dogs were atrophic. The only cells remaining were pathobiotic spermatogonia and Sertoli cells. Ova were not observed in the Graafian follicles.

The central nervous organs exhibited, as a special feature, relatively numerous pyknotic cells in the cerebral cortex.

Forty-six to Sixty-eight-Month Age Groups

The dogs in this age group died 20 to 29 days post-injection. The hemopoietic, testicular and muscular changes were similar to those of the 13-month-old group. Cysts in the hypophysis and thyroid glands

were seen. The glomerular and tubular lesions were more severe than in those younger dogs dying in a comparable period of time.

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MORTALITY IN BEAGLE DOGS FOLLOWING A SINGLE DOSE OF Co^{60} GAMMA-RADIATION

William P. Norris, Carl E. Rehfeld, Calvin M. Poole, and Frank S. Williamson

PURPOSE AND METHODS

In the investigation of radiation damage, the "single-dose $\text{LD}_{50/30}$ " is commonly used as a base for the comparison of mortality data using other methods of exposure. More explicitly, the single-dose $\text{LD}_{50/30}$ refers to that amount of ionizing radiation which, applied uniformly over the whole body during several minutes, produces death in 50% of the experimental animals within 30 days. It is well known that different strains of a given species may produce significantly different values for the single-dose $\text{LD}_{50/30}$. Thus, this quantity may be used to characterize a given animal population.

Since the beagle dog must be regarded as genetically heterozygous, it is possible that it will not respond to a given stimulus as uniformly as do more completely inbred laboratory species. Furthermore, in long-term studies, there is always the possibility of genetic or physiologic drift which may cause systematic alterations in this quantity.

Thus, a periodic reevaluation of the single-dose $\text{LD}_{50/30}$ should be a valuable check on the stability of this experimental population with chronologic time.

Dogs, suspended in canvas slings, were placed at a constant distance from the Co^{60} γ -ray source. They were given exposure doses, measured in air at the midline distance, ranging from 275 to 365 R. They were rotated 180° at the midpoint of exposure to produce a more uniform distribution of ionizing energy.

Following exposure, they were returned to their regular kennels and followed clinically and hematologically. Animals that failed to survive were necropsied and their tissues examined by histologic methods.

PROGRESS REPORT

The results from the most recent exposures are combined with earlier data and presented in Table 46. A probit least squares fit of these data produced a value for the $\text{LD}_{50/30}$ of $310.4 \pm \sigma = 2.6$ R. This least squares fit and the data points are shown in Figure 67.

The average thickness of these dogs, at the level of the last rib, was about 14 cm. From the results of Sinclair⁽¹⁾ with ferrous sulfate dosimeters, the exposure dose may be converted to average absorbed dose in rads using a factor of 0.78. Thus, the $\text{LD}_{50/30}$ in these animals may also be expressed as $242 \pm \sigma = 2.0$ rads. A somewhat better estimate may result from consideration of the actual body sizes and weights of the individual animals. Measurements using LiF dosimeters taped to the sides of the dogs during the exposures were in good agreement with Sinclair's results using ferrous sulfate. These data will be presented in more detail at a later date.

Visual observations were made on all the exposed animals daily for evidence of illness. In addition, rectal temperatures were taken on as near a daily basis as possible for all exposure groups except the 350 R group, on which only random temperatures were recorded. Due to personnel limitations, there were a few omissions in data collection; however, adequate temperature curves were obtained. Temperatures were recorded at approximately the same hour each day and with as little handling of the animals as possible.

Those animals which died exhibited severe signs of illness for periods ranging from 3 to 8 days prior to death. The clinical syndrome usually included loss of

TABLE 46. MORTALITY IN DOGS FOLLOWING SINGLE, BRIEF EXPOSURES TO Co⁶⁰ GAMMA RADIATION

Ex- posure, R	Average absorbed dose, rads	Dog No.	Date irradiated	Survival, days	Mortality
275	214	801	3-15-65	—	1/8 (12.5%)
		802		—	
		807		—	
		811		29	
		818		—	
		823		—	
		830		—	
		833		—	
Average 29					
300	234	803	3-15-65	—	2/8 (25%)
		804		—	
		808		28	
		812		—	
		819		—	
		826		—	
		831		—	
		834		19	
Average 23					
325	254	245	8-22-61	18	5/19 (26.3%)
		250		—	
		251		24	
		252	10-4-61	23	
		176		—	
		177		—	
		178	10-4-61	—	
		179		—	
		188		—	
		189	3-15-65	—	
		191		—	
		192		19	
		805		—	
		806		—	
		809		—	
		817		—	
		821		28	
		828		—	
		832		—	
		Average 22			
340	265	846	6-8-65	20	7/8 (87.5%)
		848		20	
		850		24	
		853		21	
		857		—	
		859		21	
		863		16	
		864		15	
Average 19					
350	273	783	2-18-65	18	9/9 (100%)
		784		15	
		785		15	
		787		17	
		788		17	
		790		16	
		791		22	
		792		19	
		793		17	
Average 17					
365	285	847	6-8-65	18	9/9 (100%)
		849		17	
		851		22	
		855		16	
		858		17	
		860		17	
		862		14	
		865		15	
		866		19	
Average 17

FIG. 67.—Probit plot of mortality following single doses of whole body radiation in dogs against exposure dose.

appetite, lethargy, vomiting, diarrhea, dehydration, elevated temperature, severe depression and terminal coma. Early in the course of clinical illness, the tonsils and pharyngeal mucosa become inflamed and necrotic, and small hemorrhages occurred in the oral mucous membranes. In addition, most of the terminal cases exhibited varying degrees of gross blood loss in the feces and vomitus. Those animals which survived exhibited less severe signs of illness; the severity appeared to be dose related.

The rectal temperatures revealed a great deal of individual variation in the base-line data. There was a general tendency toward temperatures which were higher than is generally considered normal, as well as individual daily variation. In the absence of other findings, it was assumed that these variations were physiologic changes due to excitement from being handled while temperatures were being taken. Because of these "normal" variations it is difficult to establish average temperatures or temperature ranges that can be considered the result of radiation-induced pathology. However, for purpose of general compari-

TABLE 47. SUMMARY OF TEMPERATURE RESPONSE IN DOGS SURVIVING NEAR-LETHAL DOSES OF Co⁶⁰ GAMMA RADIATION

Exposure, R	Total No. of survivors	No. of survivors exhibiting transient temperatures of 105.0° or above
340	1	1
325	6	5
300	6	3
275	7	0

son, a value of 105.0°F or above was considered a significant pathologic temperature. All the animals that died had temperatures in this range prior to death. The average length of time prior to death during which the temperature was elevated was 5.5 days with no significant differences between dose groups. The temperature response of the survivors, again

using 105.0°F as the dividing line, is shown in Table 47 and appears to reflect a dose response.

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MORTALITY IN BEAGLE DOGS FOLLOWING REPEATED, NEAR-LETHAL, SINGLE DOSES OF Co^{60} GAMMA-RADIATION

William P. Norris, Carl E. Rehfeld, and Calvin M. Poole

PURPOSE AND METHODS

This study is being conducted with the animals which survived an experiment (described earlier in this report) to determine the single-dose $\text{LD}_{50/30}$ of Co^{60} γ radiation. The dogs, previously given exposures of either 275, 300, or 325 R were allowed to recover for approximately 100 days. At this time they were all reexposed to 275 R. About 100 days later the survivors of the second exposure were given a third exposure of 275 R.

The purpose of this experiment is to obtain an estimate of the degree and rate at which dogs can repair damage caused by irradiation. The indices of repair are mortality and the hematological response—particularly of the leukocytes.

Dogs which died as a result of irradiation were necropsied, and their tissues were examined histologically. These results are not yet available.

PROGRESS REPORT

The survival of the dogs in this experiment, to date, is presented in Table 48. Following the second exposure, eight of the nineteen dogs died at an average of 25 days. Although the numbers of animals were small, it is clear that there was still residual damage from the first treatment amounting to, roughly, the equivalent of 35 R. There was no indication of differences as a result of the spread of exposure values in the first irradiation.

At this time, there have been no deaths following the third exposure. Some of the animals, at 21 days, are clearly approaching a terminal state, while others remain in a condition of apparent good health.

Eleven dogs survived the first and second Co^{60} exposures; they are treated as one group for purposes discussing the effect of multiple large-dose exposures on leukocyte numbers in venous blood. These eleven dogs obviously differ from the remainder of

those similarly treated because they did survive two exposures.

The average number of leukocytes per mm^3 of whole blood before exposure is 11,200, which agrees

TABLE 48. SURVIVAL OF DOGS GIVEN REPEATED BRIEF EXPOSURES TO NEAR-LETHAL DOSES OF Co^{60} GAMMA RADIATION

Dog No.	First exposure (3-15-65), R	Exposure to death, days	Second exposure (6-22-65), R	Exposure to death, days	Third exposure (9-15-65), R	Exposure to death, days
802	275	—	275	—	275	21
818	275	—	275	—	275	21
830	275	—	275	—	275	21
833	275	—	275	—	275	21
803	300	—	275	—	275	21
804	300	—	275	—	275	21
819	300	—	275	—	275	21
831	300	—	275	—	275	21
805	325	—	275	—	275	21
806	325	—	275	—	275	21
828	325	—	275	—	275	21
801	275	—	275	19		
807	275	—	275	29		
823	275	—	275	23		
812	300	—	275	23		
826	300	—	275	19		
809	325	—	275	27		
817	325	—	275	32		
832	325	—	275	29		
				—		
				25		
				Aver-		
				age		
811	275	29				
808	300	28				
834	300	19				
821	325	28				
		—				
		26				
		Aver-				
		age

FIG. 68.—Average total leukocyte counts per mm^3 whole venous blood from eleven dogs surviving first two Co^{60} exposures.

well with 11,800, which is the present standard for this colony. These cells were counted by means of a Coulter type aperture, pre-amplifier and a scaler; three counts were made on each sample.

The venous blood leukocytes were reduced in number from $11,000 \pm$ to $3,000/\text{mm}^3$ within a few days after the first exposure. The cells continued to diminish in number at a much slower rate during the next 2 weeks and then recovered at a steady rate for the next 5 weeks to just below the normal range. There was no significant variation in number until after the second exposure. At this time, the same pattern was followed as after the first exposure except that total reduction was somewhat greater and there was only about a 77% recovery compared to approximately 87% recovery after the first exposure. In each case, the last five average counts before reexposure were used as a basis for estimating percent recovery. Fifteen days after the third exposure the average total count was reduced to 1,614 leukocytes per mm^3 or 14% of the average before the first radiation exposure.

The pattern of depression and recovery is similar for the periods following the first two exposures. After the second exposure, the depression was greater and recovery less complete, but the time intervals were almost identical (Figure 68).

THE RESPONSE OF BEAGLE DOGS TO CONTINUOUS EXPOSURE TO Co^{60} GAMMA-RAYS. PART I

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PURPOSE AND METHODS

This study is part of an experimental comparison between the response of beagles to continuous whole-body irradiation from an external Co^{60} source and their response to injected Cs^{137} .

Thirteen beagle dogs, approximately 13 months old, were placed in the high-level γ -ray facility and exposed continuously to γ radiation from a Co^{60} source for a period of eleven consecutive days.

The exposure rates were either 300, 210, 147, 103, 72 or 50 R/day to the individual animals. Since the dogs were free to move about within the fiber-glass exposure cages, which had a floor area of 70 x 70 cm, the actual dose varied somewhat from the intended values. Each animal carried 4 LiF microdosimeters implanted subcutaneously, located dorsolaterally and ventrolaterally on the right and left sides at the level of the last rib.

The irradiation doses were delivered during 22 hours of each 24-hour day; the remaining 2 hours were required for cleaning of the cages of the dogs and observation.

Blood samples were obtained at regular intervals and examined hematologically, and for signs of septicemia. At death, each dog was necropsied and tissues were fixed and sectioned for histologic examination.

PROGRESS REPORT

All animals survived the 11-day period of exposure. Details of survival and radiation dosage are presented in Table 49. There was little difference in the survival of dogs at the three highest dose levels. Survival was prolonged as the dose level decreased, and the animals at 72 R/day died at an average of 21 days after beginning exposure. Those animals given 50 R/day recovered and showed no clinical evidence of residual damage when they were sacrificed for histologic observations (except for testicular atrophy and lack of spermatogenesis) at approximately 150 days. Two of the three control dogs were also sacrificed to provide normal histologic comparisons.

The LiF microdosimeters gave a measure of surface dose. The average of four determinations from each

animal was converted to the average absorbed dose by the factor of 0.94 determined by Sinclair.⁽¹⁾

At the time of removal from the exposure room, none of the animals was exhibiting any important clinical signs of illness, such as vomiting, diarrhea, dehydration, or external blood loss. Animals given the three highest dose rates (300, 210, 147 R/day) appeared to be less active than the others but were not lethargic. In general, the animals that died exhibited pronounced signs of illness for only 12 to 48 hours prior to death. In contrast, those animals which died following a single short exposure (LD_{50/30} experiment) exhibited severe signs of illness for periods ranging from 3 to 8 days prior to death.

Leukopenia was the outstanding feature of all hemograms obtained daily during the course of the exposure and intermittently prior to death. The rate at which the leukopenia developed varied directly with the dose rate, although variation was minor in the three highest levels of exposure.

The dose response was excellent, as viewed in the decreasing populations of neutrophils, eosinophils, lymphocytes, and erythrocytes in jugular blood. Thrombocyte depletion was more uniform and of no significance as an indication of dose rate differences. The monocytes and eosinophils were most radiation sensitive, and the lymphocytes appeared to be most radioresistant.

The lymphocytes were the only leukocytes found in every sample of blood; however, this may indicate that they have a longer life-span rather than greater resistance to radiation. Recent experimental evidence has been presented to show that lymphocytes may have a lifetime as great as an average of 530 days in man.⁽²⁾ The canine may also have a long-lived lymphocyte component.

In closed-colony breeding, as practiced in this laboratory, radiation sensitivity might vary with successive generations. The peripheral-blood response in this experiment was such that this could be repeated periodically as a test for the uniformity of the experimental dogs.

Gross Pathologic Findings

The gross pathologic findings in this group of animals were not unlike those described^(3, 4) for the Cs¹³⁷ dogs injected intravenously at the LD_{50/30} level. Hemorrhages were found in all organs and tissues with few exceptions; their number and extent varied considerably. Suffusions or hemorrhagic extravasa-

ns larger than 5 cm were limited to the subcutis, serosa of the intestine, and body cavities. Hemorrhage into the lumen of the intestine did not appear as extensive or as important as in the Cs¹³⁷-injected

TABLE 49. SURVIVAL OF BEAGLES GIVEN CONTINUOUS WHOLE-BODY γ -IRRADIATION

Nominal exposure (dose at cage center), R/day	Dog No.	LiF absorbed dose, rads	Average absorbed dose, rads	Survival from beginning of exposure, days
300	686	2380	2240	13
210	687	1725	1620	12
147	688	1223	1150	15
103	666	935	878	17
	689	760	715	22
72	667	628	590	21
	698	540	508	22
	699	531	500	24
	701	571	536	18
50	685	284	267	151 (Sacrificed)
	703	302	284	152 "
	704	276	260	154 "
	705	283	266	155 "
Control	669	—	—	370
	683	—	—	158 (Sacrificed)
	684	—	—	159 "

dogs, and hemorrhage of the intestinal wall was much more prominent in the serosa than in the lumen. Hemorrhage of the subcutis was greatest in the ventral cervical areas and was primarily associated with the site of venipuncture. Hemothorax, hemoperitoneum and hemopericardium as well as effusions of the mediastinum, intercostal muscles, diaphragm, pericardium, myocardium, lungs and visceral and parietal serosal surfaces of the peritoneum, pleural, and pericardial cavities were observed, but not consistently in each animal.

The distribution, quantity or extent of the hemorrhage did not appear related to dose or length of survival.

Changes found consistently included enlargement, edema, and diffuse hemorrhage of all lymph nodes; edema and hyperemia of the lungs with variations dependent on hypostasis; edema, diffuse hemorrhage and necrosis of the tonsils; congestion of the liver; petechiation and focal necrosis of the intestinal mucosa.

Microscopic Findings

The microscopic changes also resembled the changes found in dogs injected with Cs¹³⁷ at doses near the LD_{50/30} level.

Hemorrhage was the most consistent change and

was observed in all or nearly all tissues. The second most prominent finding was the presence of bacteria in many of the tissues of all animals. Microorganisms were demonstrated in every tissue, including such unusual locations as skeletal muscle, adrenal, thyroid, brain, pituitary, myocardium and the palpable lymph nodes in one or more animals. Other tissues were commonly and abundantly seeded with bacteria. These included the liver, lungs and various portions of the intestine in many instances. In many cases, nonexudative necrosis of the tissue was associated with the presence of the bacteria. In no instance was an inflammatory reaction or infiltration of leukocytes observed. The presence of bacteria was associated with tissue lysis but there was no cellular infiltration or aggregation.

Degenerative changes, many of which could be confused with postmortem autolysis, were present. These changes, however, were much more marked than seen in normal, nonirradiated tissues collected and processed under similar conditions. It seems justified to conclude that the greater portion of such changes were ante-mortem in origin and, therefore, of pathologic significance and that they are related to the extent and type of terminal septicemia which was the immediate cause of death.

Other microscopic findings were quite similar to lesions described as typical for acute radiation death. These included degeneration and necrosis of the gastrointestinal mucosa, marked depletion and atrophy of lymphoid tissues and aplasia of the bone marrow.

The amount of damage to the intestinal mucosa did not appear related to the dose except in the dogs at the two highest levels. In these two dogs the amount of damage to the intestinal mucosa was more severe than in the dogs in the 4 other groups in which the changes were similar. Damage of the mucosa of the intestinal wall, as in other tissues, appeared to be more directly related to the presence of bacteria than to the total radiation dose. In areas where bacteria were not observed, degenerative but not necrotic changes were present. Exceptions to the changes described in the cesium dogs were the lack of numerous thrombi and infarcts in the myocardium and myopathy of the myocardium and skeletal muscles. Varying degrees of degenerative myopathy were present in several animals; these included the deposition of a mucopolysaccharide material as seen in the cesium dogs. In a few instances, this material was within the sarcolemma, as in the cesium dogs, but more appeared to be located in the endomysium in this experiment. These changes are not regarded as specific responses since they were not uniform in dis-

tribution in any individual group nor did they show any relationship to dose or survival time.

Intravascular granular hyaline vacuolated bodies, similar to those demonstrated in the cesium dogs as well as in other irradiated animals,⁽⁵⁾ were also present in this group. The morphology was strikingly similar, but neither the origin nor the significance of this change is known.

In the sections of intestine, lymph nodes, and spleen, plasma cells were present and retained recognizable characteristics, while the lymphocytes were largely degenerate or gone. The degree of depletion of lymph follicles did not appear related to dose, except again at the two highest dose levels. Decedents in the 147, 103 and 72 R/day dose groups showed similar and less severe changes in the lymph nodes and spleen, as contrasted to the higher doses. In the three dose groups noted above, there was phagocytosis of hemosiderin by macrophages and greater numbers of cells in the lymph nodes. There was no obvious graded response within these groups.

Tissue changes in the 50 R survivals are largely restricted to the testicles of the two males where spermatogenesis was completely inhibited and the tubules atrophied. No other gross or microscopic changes were demonstrated.

One important problem is the difficulty in determining direct radiation effects, as complicated by septicemia and postmortem decomposition. The importance of infection is unquestionable because of the demonstration of colonies in sites which are unlikely areas for postmortem contamination, and because of the demonstration of antemortem bacteremia.

Blood samples were collected aseptically from the jugular vein at various intervals and cultured both in liquid and solid trypticase-soy medium. Cultures were incubated at 37°C and held for 1 month. Subcultures were made from the liquid medium 2 and 4 days after collection and at weekly intervals thereafter.

At necropsy, specimens of lung, liver, and spleen were collected and cultured on blood agar plates, eosin methylene blue agar and in fluid thioglycollate medium. Isolates from the blood and from necropsy tissues were identified by appropriate biochemical tests.

The examination of blood samples showed a bacteremia* at least 24 hours before death in the dogs irradiated at 300, 210 and 103 R/day as well as in three of the dogs at the 72 R/day dose. The dog in

* The microbiological procedures were conducted by Patricia A. Brennan.

radiated at 147 R/day was negative on the 2 days sampled, while the remaining animals at 103 and 72 R/day doses were not sampled prior to death. One of the 50 R/day dogs was positive on the 12th day in spite of the fact that all of these dogs survived.

On the basis of tissues collected at necropsy, all animals had a bacteremia at the time of death.

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THE RESPONSE OF BEAGLE DOGS TO CONTINUOUS EXPOSURE TO Co^{60} GAMMA-RAYS. PART II

William P. Norris, Carl E. Rehfeld, Calvin M. Poole, Frank S. Williamson, Thomas E. Fritz, and Robert Zaldivar

PURPOSE AND METHODS

This experiment is a continuation in the comparison of the response of beagle dogs to injected Cs^{137} and to externally applied radiation from a Co^{60} γ -ray source. In this study, 36 dogs will be maintained continuously in the γ -ray field for a period of 100 days or until death. They will be irradiated for 22 hours out of each 24-hour day.

The animals are caged in the same manner as described in Part I (above), and techniques for determination of the integrated dose to each are substantially identical. The nominal daily dose rates range from 17 to 72 R/day which brackets the area of interest relative to the response of dogs to Cs^{137} . Lethal or near-lethal doses of injected Cs^{137} subject a dog to an initial irradiation dosage estimated at 50 rads/day to the whole body. However, this latter dose is steadily decremented as the isotope is excreted.

Exposures were begun at 10:00 A.M., September 17, 1965 when the four 72 R/day and the eight 50 R/day dogs were introduced into the γ -ray field. The 35, 24.5, and 17 R/day groups (eight dogs per group) were placed in the field at 10:00 A.M. on the 18th, 19th, and 20th of September, respectively.

In addition to the primary information on mortality as a function of dose rate and integrated dosage, this study will provide data on hematologic, clinical, biochemical and pathologic changes associated with this type of exposure to ionizing radiation.

As animals die, they will be replaced by a second group of dogs which will be serially sacrificed for a detailed study of the progression of cytologic and pathologic abnormalities. At the various dose levels, tissues will be studied at comparable values of integrated dosage as shown in Table 50.

TABLE 50. SCHEDULE OF SACRIFICES OF DOGS UNDER CONTINUOUS Co^{60} GAMMA IRRADIATION

Integrated exposure to sacrifice, R	Daily exposure rate,* R/day				
2000			24.5	35	50
1400		24.5	35	50	72
980	24.5	35	50	72	
686	35	50	72		
480	50	72			
336	72				

* One dog will be sacrificed at each point indicated.

PERIODONTAL HEALTH-DISEASE STATE IN A BEAGLE HOUND COLONY

Henry M. Rosenberg, Carl E. Rehfeld, and Thomas E. Emmering**

PURPOSE AND METHODS

Beagle hounds maintained in controlled environments have been employed in studies to investigate the effects of bone-seeking and other radioisotopes at toxic levels. The evaluation of the oral health of the beagle hounds maintained in these colonies indicates that periodontal disease is enzootic. Changes in mandibular bone due to periodontal disease are similar to those produced by ionizing radiation so the presence of the first prohibits a radiographic assessment of subtle radiation damage.

The purpose of the present study is to achieve a more objective clarification of the characteristics of the periodontium in a nonirradiated beagle hound colony.

A preliminary, qualitative survey was made of 125 untreated breeding and control animals in the colony to determine the presence of calculus, gingival inflammation and tooth loss. This large population received no dental treatment prior to this survey.

An experimental sample of six female and six male beagles, which could be classified as representative animals, was selected specifically for this study. These twelve animals were then divided into two experimental groups based on their age and sex. Group I was fourteen to fifteen months of age, comprised of four females and two males. Group II was twenty-one to

twenty-two months of age and consisted of four males and two females. All dogs in the two experimental groups were treated alike in that the teeth on one side of the mouth received thorough scaling and prophylaxis while the other half of the teeth were untreated.

PROGRESS REPORT

The qualitative survey of the population sample (125) indicated calculus formation occurred as early as nine months of age. Calculus formation was concomitant with gingival inflammation. After twenty-six months of age heavy calculus formation was observed in 90% of the sample. After twenty-six months of age, the manifestations of true periodontal disease were observed. Extractions were necessary in twenty-three animals of the observed population, which ranged in age from 1065 to 4145 days.

In the experimental groups, there was a rapid recovery of the gingival tissue to a healthy state after calculus removal on the treated side. The animals, when observed 5 to 6 weeks post-treatment, showed an accumulation of calculus and soft debris in a pattern similar to the original condition.

Observations, 19 to 26 weeks post-treatment, showed a similar pattern of calculus deposition and gingival inflammation on both the treated and control sides. No differentiation could be made between the oral health status of the treated and control sides of the jaws.

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EXCHANGE SKIN GRAFT HISTOCOMPATIBILITY STUDY

*Carl E. Rehfeld and William J. Hester**

PURPOSE AND METHODS

Studies in the toxicity and metabolism of radio-nuclides are being made with a relatively small number of dogs, so uniformity in these animals is desirable in order to reduce the range of responses. Inbreeding is the one method which can be used to obtain uniform animals, so this practice has been followed continuously with one part of the colony. The inbreeding program began with four lines of beagles, but only two lines survive. One of these lines, the A

line, has reproduced very well; many of them have been used experimentally, and there are now 92 living members of the line. There are only 17 living members in the B line; however, they are vigorous and this line should increase within the next few years. The inbreeding coefficient for these inbred animals ranges up to 0.64; this is equivalent to approximately five generations of sibling mating. These inbred beagles have attracted the attention of investigators in other laboratories.

The degree of histocompatibility which may have developed as a result of inbreeding to date was tested

* Peter Bent Brigham Hospital, Boston, Massachusetts.

by skin grafting. This was a collaborative study with a group at the Peter Bent Brigham Hospital interested in organ transplantation.

PROGRESS REPORT

The first phase of this study has been completed. Full-thickness skin grafts were exchanged between 14 pairs of A line inbred dogs, 3 pairs of B line inbred dogs, and 3 pairs of unrelated controls. An additional four inbred dogs were used for autologous skin graft growth observation as a check on surgical technic.

The inbreeding coefficients of the inbred dogs varied from 0.45 to 0.64 where a coefficient of 1.00 represents a hypothetical state in which all allelic genes are homozygous. With only one exception, each dog of a matched pair had identical inbreeding coefficients, and they were usually siblings of the same sex.

Visual observations, photographs and biopsy specimens of the skin grafts were made or taken at suitable intervals. Histological examination of the biopsy preparations is not complete; however, visual impressions

of the grafts in organic and degenerative phases are utilized for a preliminary evaluation of graft acceptance.

Unrelated dogs usually reject skin grafts in 9 to 12 days, according to prior experience. The average time for rejection of skin grafts in Argonne unrelated dogs was 11.5 days with a range of from 11 to 12 days. The average rejection time for the A line inbreds was 18 days with a range of from 12 to 26 days. The B line was exceptional in that its average rejection time was 27.6 days with a range of from 19 to 50 days.

Skin graft acceptance was definitely prolonged in the inbred dogs, particularly in the B line. In most instances, there was a marked difference in length of acceptance time between pairs; one usually rejecting from 3 to 7 days sooner than its test mate, with as much as 25 days difference in rejection time occurring in one pair.

Other investigators participating in this study are Dr. Francis Moore and Dr. Gustav Damen of Peter Bent Brigham Hospital and Dr. William Norris and Dr. Calvin Poole of Argonne National Laboratory.

GROWTH OF BEAGLE DOGS

William P. Norris, Harry Auerbach, and Merlin H. Dipert

PURPOSE AND METHODS

The object of this study was to define the mean growth pattern in this colony of beagles so that a program of selection could be instituted to identify any animal which did not conform within predetermined limits. The parameter studied was the weight of the dogs, although other measures could be used in a similar manner. The attempt to define a mean growth pattern and its variance had two main purposes. In the first place, it would permit, by exclusion of dogs outside the limits, the development of a more homogeneous population of beagles. Exclusion of unduly large or small dogs, at an early age, which predictably would be undesirable for future use, would increase the efficiency of the program in many ways.

The second purpose arose out of the large numbers of dogs under study and the need for an efficient method of close control. All of the weights are recorded on computer tape and each set of new weights periodically updates the tape. A mean-weight growth pattern and its variance, once programmed on the computer, could be used to review the updated tapes and indicate which dogs, of any age, were for

some reason becoming too heavy or underweight. Such automatically selected dogs could be examined by the staff for possible reasons for this undue change in weight.

PROGRESS REPORT

A good fit for the first-year weight growth of beagles was obtained by use of a Gompertzian solution. In careful study of individual-dog growth plots, it became apparent that deviations were occurring in the weight pattern which were not random deviations around a mean but represented changes due to outside stimuli. Correlations of weight records with the dogs' complete kennel history revealed several procedures which affect the dogs' growth pattern.

Basically, these beagles live a relatively isolated existence in their kennels—housed with one other dog and infrequently taken out of the pen. The first major affecter of dog weight was the relationships between kennel-mates. In most, if not all cases, one dog became dominant and the other submissive. While the dogs were on measured feedings (before February, 1964) it could be shown that dominance or

submissiveness was reflected directly by the weight data. At maturity (250 days and older), the submissive dog typically showed either a failure to gain or an actual decline in weight. This is graphically illustrated when dogs on measured feedings were placed on ad lib. feedings. A dramatic rise in weight was shown by many submissive dogs, followed by a stabilization to a gradual gain in weight.

Other outside stimuli, in the course of normal animal care, also produced systematic changes in weight, some of which were still evident after several weeks. Some of these are:

1. Removal of a dog from the pen for physical examinations, teeth cleaning, anesthesia or hemogram.
2. Transfer to another kennel or change in pen mate produced losses of weight or failure to gain in the succeeding weeks until a stabilization occurred and the dog started to gain weight again in a normal fashion.

3. Vaccination or treatment for parasites frequently produced disturbances in weight growth for several weeks. A change in vaccine in the last 2 years has been shown to minimize this type of disturbance.

4. It was shown that removal of dogs to an experimental setup with extensive physical examinations and blood studies frequently produced a gradual loss in weight before the experimental procedure was instituted.

In every case, the effect of these outside stimuli was slowly dissipated, and after a period the normal weight-growth pattern returned. The length of such acclimatization periods is under study so that experimental procedures may be programmed after a return to a normal weight pattern.

Due to the necessity for isolation of the dogs, preservation of this isolation while studying the dogs is imperative. Methods of telemetering or indirect measurement are under study.

EMPLOYMENT OF TRANSPARENT COLOR POSITIVE FILM IN ROENTGENOGRAPHY

Carl E. Rehfeld, Frank O. Bellinger, and Michael W. Morgan**

PURPOSE AND METHODS

The use of Ektachrome film for color radiography was reported in 1961 by N. S. Beyer*. Its value has been demonstrated for the examination of various metal components. This type of film is being investigated for use in the study of skeletal changes with emphasis on the dog in radiation toxicity studies.

PROGRESS REPORT

The x-ray exposure necessary to produce a useful color film is 12 to 15 times that required for a satisfactory image with conventional no-screen x-ray film. The development of color film is a multistep process in which development time and solution temperature must be carefully controlled. The developed film varies from blue to green of the color spectrum with images being dark or positive rather than negative as in conventional x-ray pictures.

* Special Materials and Services Division.

The outstanding feature of the finished color film is the three-dimensional effect of the image. It is this quality which permits registration of several layers of tissues simultaneously. In conventional film the absorption and scattering of the photons by various superimposed layers of tissue results in a serious loss of detail in the final image. Of nearly equal value is the great range of sensitivity shown by the color film in which details of hair and skin can be seen in the same exposure with trabecular patterns of bone. The three-dimensional effect and wide range of sensitivity may be due to differential sensitivity of the emulsion layers in color film.

Color radiography is practical for the examination of various tissues in the live animal, if the amount of exposure is not important, or at necropsy. At present, this technic is in a developmental stage for its use in dental research, where the effect of ionizing radiation on the supporting structures of the teeth is of particular interest.

A COMPUTER PROGRAM FOR DETERMINING INBREEDING COEFFICIENTS AND RELATIONSHIP

James W. Bacus, Ronald Rau, and Carl E. Rehfeld

PURPOSE AND METHODS

The experimental subjects used in this project are a colony of beagle dogs. The colony has been maintained for the past 5 years without introducing any new breeding stock. This has resulted in some amount of inbreeding and a close genetic relationship among many of the dogs.

In addition to the variable amount of inbreeding brought about by a closed colony situation, two distinct inbred lines have been developed. These lines consist of dogs propagated through continuous brother-sister, parent-offspring, and cousin matings.

In an effort to produce a quantity of animals at a suitable age for experimental purposes, it has been necessary to make matings between these inbred lines and the larger outbred population.

Since many of these dogs are being used in toxicity experiments, it is advantageous to express the amount of inbreeding as a numerical coefficient for each dog. It is also desirable to assess the relationship between two given dogs, or a given dog and a group of experimental dogs, by using a numerical value.

PROGRESS REPORT

The method chosen to compute these values was one originally devised by Wright in 1921,⁽¹⁾ based upon the theory of path coefficients. This method is essentially that of giving weighted values to the influence of ancestors common to both parents of the animal in question. These values depend upon how frequently the common ancestors appear in the pedigree and how many generations they are removed from the animals in question. The value obtained is an expression of a decrease in heterozygosity due to many allelic genes being homozygous by descent from the same common ancestor, or ancestors. The amount of inbreeding involved per animal is expressed as a coefficient ranging from 0.0000 to 0.9999, with a larger value representing a more inbred animal.

A value for the relationship between individuals can be obtained by appropriate calculations that apply the same theory. The latter value is termed the coefficient of relationship and the former, the coefficient of inbreeding.

These two terms are more precisely defined by the following formulas:

$$F_X = \frac{1}{2} \sum [(\frac{1}{2})^{n+n'}(1 + F_A)] \quad (1)$$

$$R_{BC} = \frac{\sum [(\frac{1}{2})^{n+n'}(1 + F_A)]}{\sqrt{[(1 + F_B)(1 + F_C)]}} \quad (2)$$

Where F_X is the inbreeding coefficient of individual X , with a common ancestor F_A , n generations removed from the dam of X and n' generations removed from the sire of X . R_{BC} is the coefficient of relationship between two individuals B and C . F_A is a common ancestor n generations removed from B and n' generations removed from C . F_B and F_C denote the inbreeding coefficients of B and C , respectively.

Since there are a large number of dogs in the colony and the patterns of inbreeding and relationship are very complex, it was deemed necessary to program the computations involved and use a computer for compiling inbreeding coefficients and relationships.

A program was devised that is general enough to evaluate any group of individuals and determine the amount of inbreeding involved for each individual in the group. As the coefficients are obtained for each dog in question, they are automatically recorded and can be immediately used for computations involving that dog itself as a common ancestor. This process of automatically updating the records in a population under consideration is an advantage because it provides coefficients for all members of a group that has existed over a long period of time without the necessity of manually revising the input data.

The essential information required by the program is simply a list of the dam and the sire of each animal in the group under consideration. It is also necessary, as currently programmed, to know the date of birth, or order of birth, and the sex of each animal.

We are now designing a modified version of the present program that will calculate the coefficient of relationship between individuals.

A computer program of this nature can have a wide application in the fields of animal husbandry and livestock production. It is also a valuable aid in assessing genetic trends in experimental populations similar to those in this dog colony.

We appreciate the cooperation of Jeanne A. Pagels and Merlin H. Dipert of the computer section.

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Mammalian Genetics

INDUCED MUTATION RATE FOR SEX-LINKED RECESSIVE LETHALS AND VISIBLE AUTOSOMAL GENES IN THE MOUSE

Douglas Grahn, Frank A. Verley, Wayne P. Leslie,
Katherine F. Hamilton, and Bernard Slaughter

PURPOSE AND METHODS

An earlier report discussed the feasibility of carrying out a mutation rate study for sex-linked lethals.⁽¹⁾ In the interim, careful genetic study of the sex-linked mutation "tortoise" has led to the conclusion that it can be used as a marker gene in a test system designed to measure the radiation-induced mutation rate for sex-linked recessive lethals. Females carrying the sex-linked mutation will be mated with irradiated and normal males to produce F₁ *To* females

that will then be outcrossed to produce an F₂ generation that uses sex ratio as the end point to test for segregation of any new lethal. Linkage analyses for the location of presumed lethals will require outcrossing to additional marker stocks.

Mating System

The mating system involves reciprocal crosses between animals derived from the A/Jax and C57BL/6 strains and is as follows:

* Genetic Marker Stock: *a^a*—extreme nonagouti, *b*—brown, *bbl*—belt, *ln*—leaden, *p*—pink eye, *wa-2*—waved.

TABLE 51. SUMMARY OF DATA

Treatment	Genotype	No. of gametes tested		Autosomal mutants <i>A b c bl ln p wa 2</i>	No. of X chromo- somes at test	Sex-linked recessive lethals†		
		<i>F</i> ₁ *	<i>F</i> ₂ †			Suspects	Rejects	Unknown
Control	<i>To</i> /+	60	41	-----	41	2	10	29
	+/+	175	57	-----	-----	-----	-----	-----
Irradiated	<i>To</i> /+	51	28	-----	28	3	12	13
	+/+	125	37	-----	-----	-----	-----	-----

* Number of gametes screening for mutation to *A*, *b* and *c* from mating 1a only.

† Number of gametes screening for mutation to *bl*, *ln*, *p* and *wa-2* from matings 2a and 2b.

‡ Suspects are females with 2 or more litters and not enough male offspring to disqualify them as carriers of sex-linked lethals. Rejects are females with enough male offspring to disqualify them as carriers of sex-linked lethals. Unknown are females with insufficient offspring to qualify as suspect or reject.

Autosomal Genes

The *F*₁ generation from mating 1a will test for back mutation at the *a* locus and forward mutation at the *B* and *C* loci. The *F*₁ progeny of mating 1b will test for mutation at the *a* locus only. The *F*₂ generation from matings 2a and 2b will normally segregate 1:1 for brown and black and will manifest none of the marker stock traits unless the *F*₁ ♀ parent inherited the mutation. A ratio of 1:1 for normal to marker will then be observed among the *F*₂ progeny although there is an unavoidable complication. If the *F*₁ parent inherits a viable deficiency, then the *F*₂ progeny will segregate 1:1 for normal to marker trait. It is assumed that the occurrence of viable deficiency is a low frequency event and will not bias the estimate for autosomal mutations. A minimum of 10 offspring will be observed, since the probability of not seeing a new mutation will be 0.001 with that family size and a 1:1 segregation expected.

Sex-linked Recessive Lethals

If a new lethal is not induced, the expected sex ratio at birth for the *F*₂ generation (case i) will be 1.7:1* for tortoise females and approximately 1:1 for normal females. The case of an induced lethal at a map distance allowing random recombination is statistically the worst situation. Tortoise matings will then yield a minimum sex ratio of 3.4:1 if the new lethal has no viability effect (case ii). For more closely linked lethals the sex ratio widens rapidly. A recombination value of 25%, for example, gives a sex ratio of 8:1.

Statistical Implications

*F*₁ females that are mated will be assumed carriers of new lethals. Specifically, Type I error (α) involves the probability of discarding true lethals and Type II error (β) is the percentage of nonlethal

carrying females that are retained. The sex ratio on which the computations are based is subject to change if this is indicated by the data collected on the control matings. Sex ratio tests will be based on number of progeny ranging from 10–30. A mating may be rejected when the number of males exceeds the rejection point ($\alpha = 0.10$) for the observed number of offspring. If the mating is not rejected by the time 30 young are born, a progeny test for the presence of sex-linked lethals will be conducted. An α of 10% is selected because only 8.4% (β) false clues will be retained for progeny testing. The computations are based on the assumption that mutations are evenly distributed over the chromosome and use the swept radius concept.⁽²⁾ Reproductive performance trials indicate that *F*₁ *To* females are capable of producing 40–50 young. The efficiency of the test system improves with increased sample size. For experimental design purposes, the present estimates for spontaneous and induced sex-linked recessive lethal mutation rates were used to compute the 95% confidence intervals on the expected mutation rate. The interpretation of the results is that 3000–4000 X chromosomes each from the irradiated and control lines must be tested to get a significant difference between the expected spontaneous and induced mutation rates.

PROGRESS REPORT

Unsatisfactory reproductive performance by the test stock animals has required the following changes:

- Reduction of partial body x-irradiation dose in males from 600 R to 500 R.
- Maintenance of the tortoise stock lines in a hybrid background by alternately crossing with two unrelated lines.
- Fertility testing of all males prior to use in the study.

A first summary of available data is given in Table 51. No evidence for spontaneous or induced mutations to sex-linked recessive lethal or visible autosomal genes

* Reduced viability of *To* + ♀'s modifies the expected 2:1 ratio in test matings to the observed 1.7:1.

is seen, but the probability that mutations will occur in samples of these sizes is quite low.

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THE GENETIC EFFECTS OF A SEX-LINKED LETHAL GENE ON THE FITNESS OF CARRIER FEMALES

Frank A. Verley, Katherine F. Hamilton, Douglas Grahn, and Bernard Slaughter

PURPOSE AND METHODS

Data on the lifetime reproductive performance of "tortoise" and normal hybrid females were collected in order to determine the feasibility of using mutant females in a long-term study.⁽¹⁾

PROGRESS REPORT

Among the pairs of animals selected as parents, the incidence of sterility, mean number of litters produced, mean interval between litters, and mean duration of useful reproductive life are approximately equal for tortoise mutant and normal females. Tortoise mutant females when compared with their contemporary normal females show a reduction in litter size at birth and weaning of 33% and 55% respectively. Among the offspring of tortoise mutant females sex ratio at birth was 38% males (33% expected) and sex ratio at weaning was 40% males. The sex ratio at birth and weaning among the offspring of normal females was 50% and 49% males respectively.

Embryonic Mortality

Tortoise mutant and normal females were sacrificed during gestation in an attempt to understand the observed reduction in litter size at birth, the sex ratio changes, the stage of development at which the mutant is acting, and the systems which are affected. Females were sacrificed daily at different stages of gestation (10.5-17.5 days) in order to locate the dead embryos close to the termination of life. This facilitates handling and classification (Table 52).

The observed 11.3% difference in postimplantation loss among tortoise mutant females does not account

for the 33% reduction in litter size at birth. This discrepancy may be the result of using the average percentage loss for the age range at the time of sacrifice. The estimate of the regression of loss on day of sacrifice will give a more precise evaluation of the association between the variables.

If the estimated percentage postimplantation loss is unbiased, then the data suggest that the sex-linked recessive mutant is also effecting preimplantation losses. Information on the sex of dead embryos will aid in explaining the sex ratio shift from the expected 2:1 to 1.7:1.

Survival

Tortoise mutant and normal litter mates were checked at least once daily from birth to weaning at about 28 days. Age-specific mortality rate curves for tortoise mutant females, normal females, and males are shown in Figure 69. There is a distinct increase in mortality among tortoise mutant females. The maximum mortality occurs at 18-21 days postpartum and this coincides with the time of natural weaning. This prompted clinical tests for starvation among the tortoise mutant animals. All tests were negative. Beyond 30 days the mortality rates are approximately equal for tortoise mutant and normal females.

Body Weight

The mean body weight of tortoise mutant females is consistently lower than the mean body weight of normal females over a range from 0 to 175 days. These were not repeated measurements on the same group of animals.

*Skeletal Variations**

Pleiotropism of the tortoise mutant gene is further evidenced by visible hind and fore limb involvement. The extent of manifestation varies, but usually

* Radiographic evaluation of the skeletons was performed by C. Poole and T. Fritz.

TABLE 52. EMBRYONIC FINDINGS

No. of animals sacrificed	Genotype	Mean implants per pregnancy	% Postimplantation loss
51	Mutant	8.7	19.1
33	Normal	8.8	7.8

posterior region is more commonly and severely affected. The hind legs are weakened, they are abnormally abducted with the lateral aspects making contact with the ground, and locomotion is impaired. In severe cases fore and rear limbs are incoordinated and the animals move by dragging on their bellies. These severe cases have a frequency of less than five per cent and usually die.

Radiographic evaluation of the severely affected tortoise mutants and normal littermates have indicated the following skeletal differences: The acetabuli appear shallow, the femoral heads appear to subluxate, the posterior aspect of the pelvis (the ischii) appear to deviate laterally, and the tuber ischii are more pronounced.

Positioning and method of restraint may have effected some of the variations but there is a suggestion of abnormal pelvic development.

Radiographic evaluation of unaffected tortoise mutant and normal littermates indicate no pronounced skeletal differences.

Radiosensitivity

Data on the mean day of death and percent survival after single exposure to whole-body irradiation indicate that tortoise mutant females are not significantly more sensitive to radiation than their normal controls.

CONCLUSION

The data suggest that the sex-linked recessive mutant tortoise reduces mean litter size and body weight, increases pre- and early postnatal losses and effects skeletal changes. However, the over-all reproductive performance of the females qualifies them for use in long-term breeding studies.

PRELIMINARY INVESTIGATION OF THE INCIDENCE OF ATAPETAL BEAGLES IN A LARGE COLONY

Carl E. Rehfeld and Douglas Grah

PURPOSE AND METHODS

Most carnivores, including the Canidae, have a tapetum lucidum in the ocular fundus which may be easily seen with the aid of an ophthalmoscope. The tapetum is triangular in shape with the base of the angle being located at a level with the optic disc. It is a membrane which develops between the pigmented epithelium and the choroid, usually by the 4th or 5th week after birth, but it may not appear

FIG. 69.—Age-specific mortality rates for tortoise and non-tortoise mice between birth and weaning.

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until nearly four months of age. Since this membrane reflects light and is located behind the retina, it must serve the purpose of intensifying a visual image in diminished light.

A canine eye, which lacks the tapetum, is quite similar anatomically to the human eye. Thus, atapetal dogs may be superior to rabbits or other animals which do not develop a tapetum, in the study of radiation-induced eye lesions. For this reason, a study of the inheritance of this characteristic has

been initiated. No reference to the atapetal characteristic or its mode of inheritance has been found in a preliminary search of the literature.

This study is part of a more comprehensive evaluation of the genetic status of the dog colony developed for the study of the Toxicity and Metabolism of Radionuclides.⁽¹⁾ Twenty-five atapetal beagles have been identified within this population of about 500 living animals. Inbreeding is practiced in a portion of this colony, while it is incidental in the remainder where the atapetal dogs appeared. When carriers of the atapetal factor were recognized, they were mated again with carriers to augment the number of atapetal dogs.

All of the atapetal dogs have been sired by three normal males that can be identified as carriers of the trait. One of the males is the sire of the other two who are half-brothers born of dams that are also related.

PROGRESS REPORT

Six normal females and one atapetal female have each produced one or more atapetal offspring. Five of these females are related to the three carrier sires but the other two females, a mother and daughter, are not related to the three carrier males or the other five females assumed to be carriers on the basis of their breeding performance.

The carrier males have produced a total of 120 progeny which could be examined for the atapetal characteristic and the same summation for the six carrier females totals 114. Among matings that produced one or more atapetal dogs there were 76 offspring and 20 were atapetal. The ratio $20/76 = 26\%$, is a close approximation to the expected value of 25% for the mating of two carrier animals heterozygous for a recessive trait. On the simple assumption that a single recessive gene is responsible for the atapetal character, the mating of one atapetal female, presumably homozygous recessive, with a presumed heterozygote male should result in the appearance of atapetal in 50% of the offspring. The observed result is 5 out of 7 being atapetal, which is not significantly different from 1:1.

Additional matings are planned for a more definitive test of the hypothesis that the atapetal trait is due to a single recessive gene. The results observed to date could also be attributed to a multigenic trait undergoing progressive inbreeding fixation; a sufficient number of unrelated dogs remain in the colony to permit a test of the simpler genetic hypothesis.

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ACATALASEMIC AND HYPOCATALASEMIC MUTANT MICE. I. FINAL STATUS OF THE SEARCH FOR MUTANTS

Robert N. Feinstien, Judith B. Howard, and Joann T. Braun

PURPOSE AND METHODS

In the Division's Annual Report for 1964,⁽¹⁾ we described a search for acatalasemic and hypocatalasemic mouse mutants. At that time the large-scale screening of mice was completed, but we were not able to define the results genetically. The mutant lines obtained are briefly described below.

PROGRESS REPORT

Five true genetic aberrant lines have been established. Mutant line 1 (M-1) most nearly resembles the human acatalasemic individual. The homozygote has a blood catalase activity of 1-2% of normal; the heterozygote, about 50%.

The other four (M-2, M-3, M-4, and M-6), though they were originally established from separate, unre-

lated individuals, and though they are to be considered as distinct and individual lines, may here be listed together, for convenience. Blood catalase of the homozygotes of these lines is 10-20% of normal; of the heterozygotes, 65-75% of normal.

Two other lines, designated M-5 and M-7, have been extensively backcrossed and inbred and regularly exhibit blood catalase activity approximately 70% of normal. It has not been possible, however, to identify these animals as heterozygotes or homozygotes, nor has it been possible to produce progeny of significantly lower blood catalase activity, comparable to M-2, M-3, M-4, and M-6. It is at present considered that M-5 and M-7 represent, not catalase mutants, but modifications in the complex of factors that undoubtedly modulate the expression of catalase activity. We are still making an attempt to incorporate M-5 or M-7 catalase-lower-

ing property into homozygotes of the established mutants, in an attempt to obtain even lower blood catalase values.

The five definite mutants have all been found to be alleles. This was determined by crossing all the possible combinations of homozygotes, then crossing each F_1 generation *inter se*. The resulting F_2 generation invariably showed blood catalase activity within the range of the two original homozygotes. Had any

of the mutations been at *different* loci, one would have expected a wide range of catalase in the F_2 generation, from lower than either original homozygote, to completely normal levels.

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ACATALEMIC AND HYPOCATALEMIC MUTANT MICE. II. INVESTIGATIONS INTO THE IDENTITY OR NONIDENTITY OF THE MUTANT ERYTHROCYTE CATALASES

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

PURPOSE AND METHODS

The preceding article describes five lines of mice mutant with respect to blood catalase. The homozygote of the first of these lines (M-1) shows a blood catalase activity 1-2% of normal; the homozygotes of the other four lines (M-2, M-3, M-4, and M-6) have 10-20% of normal activity. A basic question that arises is: Do the mutant mice simply produce a lesser amount of the same molecular species as normals, or is the catalase molecule of mutant mice actually a different, perhaps less effective, species? Actually, of course, both could occur simultaneously; there could be qualitative *and* quantitative changes in catalase biosynthesis. We have been measuring the sensitivity of the catalase of the various bloods to a variety of chemical and physical agents *in vitro*, as a means of observing qualitative differences in the various mutant catalases.

PROGRESS REPORT

Because of its extremely low catalase activity, M-1 blood presents special problems and is not included in the present findings. Blood catalase from the other four mutant lines has been compared to normal blood catalase in the following respects: thermostability; inhibition by azide, hydroxylamine, and aminotriazole; and denaturation by urea and formamide. In every one of these respects, normal blood catalase dif-

fers from all the mutants. Normal blood catalase is slightly but significantly more sensitive than that of the four mutants to inhibition by azide or hydroxylamine, both of which presumably attack the heme moiety of the catalase molecule. Normal blood catalase is appreciably, sometimes strikingly, less sensitive than the mutants to inhibition by urea, formamide, aminotriazole, or heat, the actions of which are presumably all on the apoprotein. Other points of significant difference are as follows:

- 1) Sensitivity to aminotriazole: M-2 and M-4 differ from M-3 and M-6.
- 2) Sensitivity to hydroxylamine: M-4 differs from M-2, M-3 and M-6.
- 3) Sensitivity to urea: M-2 differs from M-3, M-4, and M-6.

CONCLUSIONS

The erythrocyte catalases of mutants M-2, M-3, M-4, and M-6 all represent molecular species distinguishable from normal blood catalase. In addition, the catalases of M-2 and M-4 differ from each other and from M-3 and M-6, which to now have shown no difference in properties. We offer the suggestion that we may have here a situation comparable to that of the known wide variety of human hemoglobin species, where molecular subunits vary in only one or a few of the constituent amino acids.

ACATALASEMIC AND HYPOCATALASEMIC MUTANT MICE. III. METABOLISM OF METHANOL AND ETHANOL

Robert N. Feinstein and Barbara Halliburton*

PURPOSE AND METHODS

The possible role of catalase in the peroxidative catabolism of alcohols has been a moot point for a long while. The literature on this question is too voluminous to be reviewed here, but it may be generalized that one school argues that essentially all alcohol metabolism is accomplished by the alcohol dehydro-

TABLE 53. "RIGHTING TIME" AFTER INTRAPERITONEAL METHANOL OR ETHANOL

Group	Mice*	Treatment	Total No. of tests	Righting time, mean $\pm$ standard deviation, minutes
1	CP	MeOH	41	35.3 $\pm$ 44.7
2	CN-1	MeOH	45	13.0 $\pm$ 21.4
3	CP	AT-MeOH	38	25.0 $\pm$ 24.3
4	CN-1	AT-MeOH	41	8.8 $\pm$ 13.6
5	CP	EtOH	37	24.0 $\pm$ 19.0
6	CN-1	EtOH	40	21.0 $\pm$ 16.7
7	CP	AT-EtOH	28	48.6 $\pm$ 27.3
8	CN-1	AT-EtOH	33	40.1 $\pm$ 25.2

Group comparisons

Groups compared	Student's <i>t</i>	Probability <i>p</i>
1 vs. 2	2.98	<0.01
3 vs. 4	3.68	<0.001
5 vs. 6	0.74	Not significant
7 vs. 8	1.27	Not significant
1 vs. 3	1.26	Not significant
2 vs. 4	1.08	Not significant
5 vs. 7	4.29	<0.001
6 vs. 8	3.57	<0.001

* CP = catalase positive (normal mice); CN-1 = catalase negative (acatalasemic or M-1 mutant).

genase system, while another school argues that catalase plays a significant role in the catabolism of at least methanol.

The development here of mutant strains of mice deficient in catalase activity suggested a fresh approach to this problem: a comparison of the rate of alcohol utilization by mice high or low in catalase, but otherwise comparable. It must be borne in mind, however, that even our M-1 mutant, the most nearly acatalasemic of our five mutant lines,⁽¹⁾ still has 1-2%

of normal blood catalase activity. In addition, M-1 mice have tissue catalase activities ranging from 8% of normal in kidney, to 38% of normal in brain. In fact, if entire animals are homogenized, it is found that the homogenate (whole-body catalase) averages 22% of normal. By the preliminary injection of the catalase inhibitor aminotriazole (AT), this figure can be reduced to 1% of normal.

Four general types of study have been applied to this problem:

1) Kinetics of disappearance of methanol from the blood stream of normal and M-1 mice, with and without prior injection of AT.

2) Metabolism of methanol by blood lysates or liver homogenates of the two lines of mice, with and without AT added to the *in vitro* system.

3) Toxicity of methanol and of ethanol to normal and M-1 mice.

4) Time required for normal or M-1 mice to recover from the acute, prostrating effects of intraperitoneal methanol or ethanol. This was measured as the time required for a 24-hour starved mouse, injected with a standard dose of methanol (6 g/kg) or ethanol (4 g/kg), to regain his feet after being injected and placed on his back. This time is designated briefly as "righting time."

PROGRESS REPORT

The first three techniques above—disappearance of methanol from the blood stream, disappearance of methanol from *in vitro* blood and liver systems, and toxicity of ethanol or methanol—may be summarized quite simply by saying that at no time, under no circumstances devised, was any significant and reproducible difference observed between normal and M-1 mice, or their tissues.

Surprisingly, however, clearly significant differences were observed in measuring the "righting time." This is indicated in Table 53, from which the following conclusions may be drawn:

1) The acatalasemic mouse rouses from the stupefying effects of methanol faster than does a normal mouse.

2) There is no difference between these two mouse lines with regard to ethanol anesthetization.

3) Aminotriazole does not alter the methanol effect.

4) The AT-injected mouse is narcotized longer by ethanol than is the mouse not treated with AT.

* Associated Colleges of the Midwest Semester Student from Coe College.

We have no explanation for these significant effects on narcotization time but are unable, in any other way, to demonstrate any difference in alcohol metabolism by the mutant mouse. In fact, it will be noted that the effect of the mutation on methanol narcotization is in the unexpected direction: the acatalasemic mouse, which might be expected to have some difficulty in disposing of methanol, actually rouses *faster* than does the normal animal. A possible explanation might be that narcotization, or prostration,

of the mice is actually effected not by the methanol itself, but by one of its oxidation products, formaldehyde or formic acid. This explanation, however, is far from satisfactory, and the phenomenon remains, for the moment, unexplained.

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GENETIC VARIATIONS IN BENCE-JONES PROTEINS

*Allen B. Edmundson, Florence Sheber, Nanne Simonds, Betty Bangs, and Timothy Johnson**

PURPOSE AND METHODS

The emphasis in this problem is centered on the interrelations of protein structures, their biological functions, and their genetic controls. We are particularly interested in the myeloma proteins and the γ -globulins. Unlike the hemoglobins, myoglobins and pancreatic enzymes of any one species, the γ -globulins vary both among individuals and within the same individual. Because of this heterogeneity, purification is difficult, and crystallization has not yet proved possible. Therefore, the γ -globulins are not suitable for x-ray analysis, and the molecules are too large for the application of conventional sequence studies by chemical methods.

These difficulties have led to the development of alternative procedures for investigation of the structures of this class of proteins. For example, R. R. Porter⁽¹⁾ and his colleagues in London found that γ -globulin could be enzymatically cleaved into three large, but more manageable fragments, one of which (the Fc fragment) can be crystallized. More indirect information has been obtained by the use of proteins from patients who have multiple myeloma. Proteins with structural characteristics similar to those of the γ -globulins are present in the serum in large quantities. They are much more homogeneous than the γ -globulins and consequently are relatively easy to purify.

Both the myeloma proteins and the 7s γ -globulins are believed to be composed of two light and two heavy chains. To simplify the structural problems, the linkages between the light and heavy chains can be cleaved, but the fractionation of the liberated subunits is difficult. In many cases of multiple myeloma, the light chains are produced in excess and are excreted

into the urine. These are named Bence-Jones proteins after their discoverer. Like the myeloma proteins, the Bence-Jones proteins can be readily purified in large quantities. In still other patients with "heavy chain" disease, fragments of the heavy chain are found in the serum. Initially, we decided to investigate the least complex of this series, the Bence-Jones proteins. If successful, the study can be extended to a comparison with the myeloma proteins from the same or different patients. With the millions of proteins which can be investigated, we are trying to restrict our efforts to those which may give some insight into major biological problems. Using the Bence-Jones and myeloma proteins as test compounds, we are interested in two of these problems: 1) the genetic control of variations which permit the individual to respond to such a wide variety of antigenic stimuli, and 2) the related problem of the antigen specificity of antibodies.

The genetic control of proteins is manifest in the amino acid sequences. The three-dimensional configurations, in turn, are believed to be primarily dependent on the amino acid sequences, rather than on direct genetic mechanisms. From the studies of sequences alone, therefore, it may be possible to deduce the genetic changes necessary for the variations. This knowledge can also be applied to the problem of antigenic specificity of antibodies, but in this case the three-dimensional structures are also of importance. Unfortunately, the three-dimensional configurations can still not be predicted from the sequences, although substantial progress may be made in this direction within 5 years. Until then, it is necessary to rely upon x-ray analysis, which is at present even more difficult and expensive than sequence analysis. We hope to pursue both approaches in this laboratory for the next few years.

* Argonne Semester Student from the Associated Colleges of the Midwest.

On the chemical side, the work on the Bence-Jones proteins was initiated in February, 1965, with modifications of the methods employed by Edmundson⁽²⁾ in the determination of the sequence of myoglobin. In September, 1965, Dr. Marianne Schiffer began a program of x-ray analysis. If the Bence-Jones and myeloma proteins prove suitable for crystallography, they are logical proteins to be examined.

PROGRESS REPORT

The Bence-Jones proteins were prepared at the University of Wisconsin by Dr. Oliver Smithies, with whom we are collaborating. Using starch gel electrophoresis under a variety of conditions, Dr. Smithies found that the samples, while comparing favorably with those from other laboratories, were still contaminated with small quantities of albumin and other impurities. At Argonne, therefore, it was necessary to devise a rapid and reproducible procedure for final purification. Albumin has a higher molecular weight than the Bence-Jones proteins, and it is likely that other contaminants include fragments having lower molecular weights than the intact Bence-Jones proteins. These differences in molecular sizes were found to be sufficient to permit the removal of the contaminants by gel filtration on columns of Sephadex G-75, with acetic acid as eluent. The purified Bence-Jones proteins usually did not emerge from the columns as single components. One protein, named "Dillon" for the patient from whom it was obtained, was separated into three components. The elution volumes corresponded to those predicted for an aggregate, a dimer, and a monomer of a protein with a minimum molecular weight of about 20,000. With the exception of the cystine content, the amino acid compositions of the three components were the same.

One of us (T.J.) found that mixtures of the three forms were precipitated by 0.5% sodium dodecyl sulfate (SDS) and not redissolved until the concentration was raised to greater than 2-3%. Even at these concentrations, the dimer could not be dissociated to the monomer in appreciable amounts. Johnson found that the chemical reason for this stability was the same as that reported by Milstein^(3, 4) for his Bence-Jones proteins. There are two antigenic types assigned to this class, and the Dillon protein belongs to Type I. All Type I light chains investigated to date are probably connected to the heavy chain by a disulfide bond located at the C-terminus of the light chain. When the light chain or Bence-Jones protein is isolated, its half of the disulfide bond would exist as a free SH group if

this were stable. In the exposed position, however, it isn't stable, and it reacts with another SH group to form a different disulfide bond. In the monomer the additional SH group is supplied by the amino acid, cysteine, and in the dimer, the terminal SH groups from two protein molecules form the disulfide bond. The aggregates are probably formed by noncovalent hydrophobic interactions. Milstein and we have found that similar arguments apply to the formation of dimers of Type II proteins, but here the half-cystine is the penultimate and not the last residue in the chain.

Since Hilschmann and Craig⁽⁵⁾ and Titani, Putnam, and their coworkers⁽⁶⁾ have concentrated on sequences of Type I proteins, our program has been mainly directed at those of Type II. However, Johnson did compare the Dillon protein with those studied by Hilschmann and Craig. They reported that the C-terminal half of one protein was identical with that of a second, except for one residue. Since substantial differences were noted in the N-terminal regions, they proposed that genetic variations, other than point mutations, would be reflected only in the N-terminal halves of Type I proteins. Johnson found that the Dillon protein differed significantly from the two proteins studied by Hilschmann and Craig in amino acid composition and molecular weight, but it is not possible at present to localize the differences.

In the case of the Type II proteins, we have made substantial progress on peptide mapping of tryptic hydrolysates of one protein, called "Hulette-Way." We have very little outside data with which to compare our results, but we have demonstrated that the C-terminal 8 residues that Milstein has assigned to the sequences of several Type II proteins also are present in the Hulette-Way protein. In analogy with the Type I proteins, the differences in the Type II proteins may also be greatest at the amino ends.

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INHIBITION OF H^3 -THYMIDINE INCORPORATION IN PIG KIDNEY CELLS BY FAR-RED LIGHT

Chen-ho Chen and Solon A. Gordon

PURPOSE AND METHODS

An enhancement in the number of chromosome aberrations can be observed when pig-kidney cells are exposed to far-red light.⁽¹⁾ This effect of the far-red can be correlated with an impairment by the far-red spectrum of phosphate esterification⁽¹⁾ and mitochondrial phosphorylation of ADP.⁽²⁾ Since irradiation of the kidney cells at mid-interphase appeared to be cytologically the most effective period, we suggested⁽¹⁾ that a limitation of high energy phosphate, particularly in the constitution of the DNA moieties, might increase the probability of chromosome aberration. This report will show that H^3 -thymidine incorporation by pig kidney cells in monolayer culture is impaired by far-red light.

Pig kidney cells, ca. 5×10^4 , of a near diploid population, (loc. cit.) were subcultured at 37°C in Leighton tubes containing 2 ml Morgan's 199 medium with 5% lamb serum. Seventeen hours later, during the exponential growth phase, the culture tubes were transferred to a rotating table within a monochromator of the Withrow type and were allowed to equilibrate for 20 minutes at a temperature of 17°C. They were then exposed to various radiant densities of far-red light (cf. Reference 3).

Immediately after exposure, the medium was replaced with 2 ml fresh medium (37°C) containing H^3 -thymidine (s.a. 0.36 Ci/mM) at a concentration of 0.2

$\mu\text{Ci/ml}$, and kept at 37°C for 10.0 minutes. Following two rinses with hypotonic Hank's solution, which was then allowed to remain for 8 minutes, the cells were fixed in acetic methanol (1:3) and air dried. With the exception of far-red exposure, all manipulations involving the cells were carried on either in the dark, or when essential, under dim green light. Autoradiographs were prepared with Kodak NTB 3 Liquid Emulsion. The slides were exposed for 6 days at 4°C, developed in Kodak D19, and counterstained with hematoxylin and eosin. Grain counts were made at random on 100 labeled interphase nuclei, 50 on each of two slides from every far-red treatment. The slides used for scoring showed no more than four background grains per nuclear area.

PROGRESS REPORT

Grain numbers were found to be highest in nuclei that received no exposure to the far-red, and decreased inversely with the radiant density. The pooled average net grains scored for each radiant exposure are plotted in Figure 70. The first significant decrease in grain count occurred at an exposure of 14 millijoules $\cdot \text{cm}^{-2}$.

Since a low specific activity of tracer was used, no or little pool effect of unlabeled thymidine^(4, 5) would be expected in the H^3 -thymidine-treated cells. The differences in grain counts scored for the various radiant densities must, therefore, represent differences in rate

FIG. 70.—Effect of far-red light on the incorporation of tritiated thymidine by nuclei of pig kidney cells in monolayer culture

of uptake of thymidine into DNA.^(4, 6) Since the rate of H³-thymidine incorporation is directly correlatable to DNA content,⁽⁷⁾ the decrease of grain number in the irradiated pig kidney cells indicates a depression of DNA synthesis quantitatively related to far-red exposure. It is possible that the far-red impairment of DNA synthesis is causally related to the action of this spectral region on phosphorylation.⁽²⁾

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ON THE ENHANCEMENT BY FAR-RED LIGHT OF X-RAY INDUCED ABERRATION FREQUENCIES IN CHROMOSOMES OF PIG KIDNEY CELLS

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PURPOSE AND METHODS

Since the initiatory work of Kaufmann *et al.* with *Drosophila*, the ability of far-red light to increase the chromosome aberration frequencies induced by ionizing radiation has been investigated chiefly with plants. Several years ago we began work with animal cells *in vitro* to define the wavelengths in the visible spectrum that are effective on such cells, and to characterize the biochemical mechanisms involved. In preliminary experiments with cells of the ascites tumor,⁽¹⁾ we observed that bracketing or closely following x-irradiation with an exposure to light of ca. 750 m μ wavelength enhanced the aberration frequencies scored one day later. For several reasons ascites cells proved unsatisfactory, and we turned to cells of the pig kidney grown in monolayer.⁽²⁾ In the present report we will show that exposure of pig kidney cells to far-red light both pre- and postexposure to x-rays raises the aberration frequencies scored at various times after irradiation.

Cultures of a near diploid line of pig kidney cells⁽³⁾ were obtained from Agnes Stroud. They were cultured in monolayer at 37°C in Leighton tubes containing 2 ml of 5% lamb serum in Morgan's 199 medium. Phenol red was omitted from the medium over cells to be exposed to radiation. Cells were x-irradiated through the medium, dose rate at surface 23 R per minute. Far-red light at ca. 740 m μ was obtained from a monochromator of the Withrow type; the irradiance incident to the cells was 0.3 mW·cm⁻².

Twenty-four hours after subcultivation, Leighton tubes containing 5×10^4 cells were exposed to: 1) 270

millijoules·cm⁻² of far-red light followed within 1 minute by 100 R of x-ray; 2) 100 R of x-ray; 3) 100 R of x-ray followed by 270 millijoules·cm² of far-red light; 4) no radiation. Irradiation was at room temperature, ca. 22°C; the cells were then returned to 37°C.

Samplings were made at 2 hour intervals up to 18½ hours after x-irradiation. For cytological examination, the cells were treated with colchicine for one hour, Hank's hypotonic solution for 8 minutes, fixed in acetic methanol, air dried, and then stained with aceto-orcein. Each datum presented represents the mean of 6 preparations and the examination of 300 cells in metaphase.

During the period from subcultivation to the time of first sampling, the cells were not exposed to light other than the experimental treatment. At that time manipulations were carried out under dim green light from a safelight assembly.

PROGRESS REPORT

In samplings 2½ hours after x-irradiation, an appreciable number of cells exposed to x-rays were pycnotic; they were not scored. Figure 71 illustrates the average number of chromosome aberrations per cell at various times afterward. In all instances, aberration frequencies were highest in the first scorable samples and then decreased. The x-irradiated cells that received no far-red light consistently showed a low aberration frequency than those pre- or posttreated with far-red light. The differences in aberration rate appear also related to the sampling time, becoming in-

TABLE 54. FREQUENCY DISTRIBUTION OF CHROMOSOME ABERRATIONS INDUCED IN PIG KIDNEY CELLS BY FAR-RED AND X-IRRADIATION

	Number of breaks per cell						Total
	0	1	2	3	4	5 or more	
I FR + x-ray	143	93	37	15	6	6	300
II x-ray	177	86	21	10	3	3	300
III x-ray + FR	144	89	34	13	11	9	300

$$\chi^2 \text{ I, II, III} = 18.75; p = 0.025-0.05.$$

$$\chi^2 \text{ I vs. II} = 11.23; p = 0.025-0.05.$$

$$\chi^2 \text{ II vs. III} = 14.48; p = 0.01-0.025.$$

$$\chi^2 \text{ I vs. III} = 2.43; p = 0.75-0.90.$$

significant somewhat over 16 hours after irradiation. This implies that the present line of cells, under the conditions described, completed its first division cycle within 15 to 17 hours. The highest aberration frequency scored was for the cells sampled 4½ hours after x-irradiation; this may indicate that the pig kidney cells were relatively sensitive to x-irradiation at late interphase. In a Chinese hamster cell line, Hsu *et al.*⁽⁴⁾ found the cells most sensitive to x-radiation in the period following DNA synthesis.

Considering the photon energy at 740 mμ, we assume that the primary damage is initially identical in the three x-ray treated cell groups. Exposure to far-red light would simply alter the expression of chromosome aberrations elicited by x-irradiation. Data taken from different samples of each treatment were therefore pooled and classed according to the number of breaks per cell. Chi-square analyses of the distribution were made; the results are shown in Table 54. Significant differences were detected between the groups "x-ray alone" and either "far-red followed by x-ray" or "x-ray followed by far-red." No material difference was found between the groups x-ray followed by far-red and far-red followed by x-ray. These results indicate that

far-red light at ~740 mμ in wavelength applied either preceding or following x-irradiation increases the number of breaks per cell.

Wolff and coworkers⁽⁵⁻⁷⁾ have considered this enhancement phenomenon to be an artifact of a far-red-induced mitotic delay. In their experiments with plant root tips and microspores, they scored the aberration frequencies at various times after x-irradiation, and found the time of peak aberration frequency to be delayed by far-red. Thus, for example, in beginning the scoring 3 hours after x-irradiation of *Vicia* root tips (Reference 7, Figure 1) far-red supplement caused a decrease in aberrations until about 15 hours after irradiation. The aberration peak frequency occurred several hours later than that of x-irradiated cells alone, but was not significantly different in magnitude. In the subsequent period, the aberrations scored were greater in the population receiving the far-red supplement. They suggest that, since various portions of the cell cycle differ in their sensitivity to x-rays, the far-red induced mitotic delay can lead to apparent increases in aberration yields if cells are scored at only one time after x-irradiation. When the whole cell cycle was sampled, however, far-red was found not to affect the maximum yield of aberrations.

Several points may be made concerning the above hypothesis. First, the investigations of Wolff *et al.* employed relatively large incident energies of far-red light, 50⁽⁵⁾ and 400⁽⁷⁾ joules·cm⁻², involving 3-hour exposures either preceding or following the x-irradiation. With pig kidney cells, exposures of such duration, involving incident energies of ca. 4 joules·cm⁻², do elicit shifts in the time at which peak aberration frequencies occur (Reference 2, Figure 71), without material change in aberration magnitude. But with such cells, maximal aberration (Reference 2, Figure 72) and decreased DNA-formation⁽⁸⁾ effects of far-red per se are achieved with radiant densities on the magnitude of 0.05 to 0.1 joule·cm⁻². The aberration yield

FIG. 71.—Aberration frequencies in pig kidney cells exposed to x-rays (100 R) and far-red light (740 mμ, 270 millijoules·cm⁻² incident).

decreases with higher incident energies, becoming negligible at 4 joules·cm⁻² when the kidney cells were at 24°C. We raise the possibility that high-energy densities of the far-red may elicit mitotic shifts without causing an enhancement of aberration yield. In this respect, it may be analogous to the photomorphogenic effects of the red—far-red spectral regions upon plants, where extended exposure to one band will simulate the effects of the other, and cause morphogenic reversal.

Second, Wolff and Luippold⁽⁷⁾ stress that the far-red has no effect on the x-ray induced aberration yield when the roots of the bean were x-irradiated in G_1 , a period of uniform radiosensitivity. When irradiation was given when an appreciable number of cells were in S or G_2 , periods of nonuniform sensitivity, the mitotic shifts leading to an apparent synergism takes place. We, also, find that kidney cells, synchronized by subculture, are not cytologically responsive to the far-red in G_1 ; sensitivity begins as the cells move into S . In the accompanying paper⁽⁸⁾ we show that the far-red impairs the ability of the kidney cell to incorporate H³-thymidine. It may be suggested that the lack of sensitivity of G_1 to the far-red is simply a reflection of the absence of the locus of far-red action that is involved, DNA synthesis.

Third, and perhaps most important, are the observations in the present report that pre- or postsupplementation of the x-rays with far-red consistently increased the aberration frequencies scored over a period equal to or somewhat longer than the cell cycle. These considerations, plus those dealing with the aberrancies

occurring after irradiation with only the far-red,⁽²⁾ lead us to infer that the enhancement of chromosomal damage in kidney cells by far-red light is not simply a consequence of mitotic shifts.

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THE APPLICATION OF COMPUTERS FOR THE ANALYSIS AND PAIRING OF CHROMOSOMES

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PURPOSE AND METHODS

The automatic analysis of photomicrographs of mammalian chromosomes by computers is essential to obtain rapid information concerning chromosome abnormalities produced by ionizing radiations and chemicals and abnormalities associated with genetic diseases.

Chromosome material that requires analysis is accumulating at such an increasing rate that the need for quantitative and statistical analysis exceeds that possible by ordinary microscopic methods because of the tedious and time consuming nature of classical laboratory procedures. We have made use of the

rapid information-processing capabilities of a digital computer and a precision cathode-ray tube film scanner, CHLOE, to accommodate the demands of these studies. Earlier attempts at the computer analysis of chromosomes have been reported;^(1, 2) this report describes our most recent progress and presents illustrations of the complete pairing of chromosome complements.

The characterization and the pairing of chromosomes are achieved by the CHLOE scanning unit, which is controlled by an ASI 210 computer, and the data are analyzed by an IBM 704 computer program. The reconstruction of the chromosome figures from the digital data is done by the computer GEORGE, which is equipped with a cathode-ray tube output device. Es-

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essentially, the system requires that the chromosomes at metaphase be photographed on 35-mm film so that it can be scanned by the digitally-controlled cathode-ray tube scanner CHLOE. The measurement and shape-construction are controlled by the ASI 210 computer, and the data are processed from the numerical information, resulting in a reconstructed metaphase picture of the chromosomes with a line segment drawn between each homologous pair. The IBM 704 computer program pairs each chromosome with its corresponding mate on the basis of shape similarity. An aberrant chromosome produced by irradiation, with deletions or reciprocal translocations, cannot be paired because there is no corresponding chromosome. Such anomalies are sorted out and classified as aberrant chromosomes. Our present experimental studies include radiation-induced chromosome aberrations of pig kidney cells in tissue culture,⁽³⁾ and later, the African rat (*Mastomys natalensis*). Thus, we are concerned with the defection of chromosomes with minute changes that involve deletions or translocations which are not always detectable by eye, but that are reliably detected by such a rapid automatic and sensitive scanning-device such as CHLOE. At the present, a metaphase spread with a full complement of chromosomes, randomly arrayed, can be scanned with this system in less than two seconds, and the digital information can be stored on magnetic tape and processed in less than two minutes for each complement. The scanning system, CHLOE, and the ASI 210 computer are to be connected to the CDC 3600 computer this year, eliminating the 704 computer and the computer GEORGE. This will reduce the automated chromosome complement time to 0.1–0.2 minute.

PROGRESS REPORT

The CHLOE scanning program has achieved its goal, that is a complete pairing of all 46 chromosomes in a metaphase-spread of female *Mastomys natalensis* kidney cell (Figure 72). The figure shows the film input data (above) and the output data (below) reconstructed from the digital information with a line seg-

tute a pair. The choices are then arranged according to chromosome type (subtelocentric, metacentric, telocentric) and size from within the spread. Eighteen pairs are represented, including the sex chromosomes.

The computer pairing is based on a collection of line segments (50–100) of the chromosome so that only the edge coordinates of the shapes are measured. Recognizing the closed shapes as regions of constant density, the IBM 704 program computes the centroid of the chromosome and the first 8 central moments (through the third order) of each shape and attempts to form initial clusters of shapes for assembly into chromosomes. The method used in the program to classify chromosome shapes is based on the moment invariants as described by Hu.⁽⁴⁾ These are algebraic combinations of absolute moments that are invariant under the various subgroups of the full affine group in the plane. The particular invariants used here may be referred to as Euclidean moment invariants, because they are chosen to be invariants under the extended Euclidean group, (the group in the plane consists of translations and proper and improper rotations). The first 7 of these invariants have been used, and are formed from absolute moments through the third order. This permits each chromosome to be associated with one point in a 7-dimensional space that can be made into a metric space by introduction of a suitable metric. Moment invariants have also been used for recognition of printed letters by Alt.⁽⁵⁾

After an initial cluster is formed, the program tests all possible subclusters to identify which of them appear as chromosomes. This is done by merging trial subclusters (adding their absolute moments), computing the 7-moment invariants for the merged clusters, and accepting only the largest clusters whose representative points lie inside a specific polygonal region in the moment space. Successful clusters are stored in the computer memory in the form of 9 quantities for each cluster; these quantities are the coordinates of the centroid and the 7 invariants.

The moment space is now converted into a metric space by definition of the distance

$$D = \left| \frac{J_0 - J'_0}{J_0 + |J'_0|} + \frac{J_1 - J'_1}{J_1 + |J'_1|} + \cdots + \frac{J_6 - J'_6}{J_6 + |J'_6|} \right|$$

ment drawn from each chromosome to its homologous mate. A comparison between manual pairing (human) and the automated pairing (computer) of the chromosomes is shown as a karyotype of the animal in Figure 73. The manual pairing is done by visual examination of the chromosomes with a decision having to be made about the most likely chromosomes that consti-

where $J_0 \cdots J_6$ are the 7 moment invariants and the primed and unprimed symbols refer to the two chromosomes between which the distance is measured. This distance function is scale invariant and has the obvious property ($0 \leq D \leq 1$). In computation of the invariants, the formulas of Hu⁽⁴⁾ were modified in such a way that they cause each invariant to have the same

FIG. 72.—Top, a methaphase spread of 36 chromosomes of *Mastomys natalensis*, representing the input data for the CHLOE computer. Bottom, the output data reconstituted from the digital information together with connecting line segments illustrating the pairing obtained by the computer program.

FIG. 73.—A karyotype of the chromosomes of *Mastomys natalensis* manually paired compared with the CHLOE computer program pairing.

physical dimensions. The program also incorporates an adjustable scale factor for each of the invariants $J_1, \dots, J_6$.

An array of numbers is then formed whose elements are the distance between each pair of chromosomes in the 7-dimensional metric space. The pair with the smallest distance is reported out as a valid pair, followed by the pair with the next smallest distance, etc., until all the chromosomes are included. The monotonically-increasing sequence of distance measurements obtained

by this process is independent of the scale of the photograph and the arrangement of chromosomes in the spread. It is, therefore, an invariant property of the karyotype.

On analysis of the computer pairing versus human-manual pairing of *Mastomys natalensis* (Figure 73), it was found that *the computer was more efficient than man in selecting the correct chromosome with its homologous pair*. This information was obtained by examining the areas of the chromosome pairs that is one

of the 7 moment invariants (J_0). The computer was also more efficient in placing the right pairs together when 2 or 3 pairs of one type were individuals in a group,⁽⁶⁾ such as the 3 small metacentrics in Figure 73 (pairs 10, 11, and 12). Among the chromosomes paired by hand, three pairs of chromosomes were paired with non-homologous chromosomes (chromosome pairs 5, 16, and 17). The correct pairing was checked for accuracy against the 35-mm negative used as the input data for CHLOE. We have found that photographic prints are not completely reliable for chromosome pairing. This gives the computer an obvious advantage.

CONCLUSIONS

The most recent results obtained from the computer pairing and recognition of chromosomes are encouraging. The incorporation into the computer program of not only the recognition of chromosome shapes but of their density is being programmed. These two measurements should increase the reliability of the CHLOE method and should lead to a new and promising ap-

proach to cytogenetics and, therefore, place the subject on a more quantitative and reliable basis.

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Microbial Genetics

ABSENCE OF SEGREGATION FROM NEWLY-FORMED MUTANTS IN A STRAIN OF BACTERIA WITH REDUCED DARK REPAIR ABILITY

Herbert E. Kubitschek

PURPOSE AND METHODS

Newly-formed mutant cells failed to display a segregational division following mutation of *Escherichia coli* B/r/1, try⁻.⁽¹⁾ This result was of interest because it implied the possible failure of the Watson-Crick model of replication of DNA.⁽²⁾ Their model requires that mutation of but a single strand of DNA of a parental duplex give rise to one mutant duplex and one nonmutated duplex after replication. In order to explain the absence of segregation with the Watson-Crick model, it is necessary to assume that mutation of one of the two strands of DNA is followed by mutation of its complementary strand before cell division can occur. Mutation of both strands in this fashion could occur in the presence of DNA-repair enzymes, known to be capable of restoring sequence complementarity.

Since the Watson-Crick model of replication fails to explain the absence of segregation if repair is ruled

out, it was of interest to test for segregation in a strain of bacteria known to be incapable of repair.

PROGRESS REPORT

The kinetics of mutation to resistance to bacteriophage T5 were measured in continuous chemostat cultures of the strain of *Escherichia coli* B_{s-1}, in which repair ability is reduced or absent. Mutations were induced by the addition of acridine orange to the culture followed by a brief exposure to visible light. The most significant result, Figure 74 is the absence of a common segregational division in newly-forming mutants; a segregational division would have required the concentration of latent mutants to decrease by a factor of two during the first postmutational cell division, rather than remaining reasonably constant after irradiation.

CONCLUSIONS

These results cannot be explained by the Watson-Crick model of replication, because it requires that

FIG. 74.—Kinetics of Mutation in *E. coli* B₈₋₁. Mutation to T5 resistance was induced with acridine orange and visible light at the time shown by the arrow. Mutant frequencies are shown for phenotypically expressed mutants, open circles, and for latent mutants, filled circles. Latent mutants were measured as those cells giving rise to resistant colonies after a preliminary incubation period of three hours before exposure to bacteriophage.

mutation of a single strand of DNA lead to a segregational division in a nonrepairing strain. Instead, these results strongly suggest that a single master strand of DNA specifies the coding sequence of both newly-forming strands of DNA.

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CELL DIVISION: ANCESTRAL DEPENDENCE OF GENERATION TIMES

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PURPOSE AND METHODS

Theoretical models for the growth of cell populations have usually assumed that the generation time of each cell, from "birth" by fission to "death" by fission, is independent of the generation time of all other cells. Several experimental studies⁽¹⁻³⁾ have shown that this assumption is false because sister cell generation times are highly correlated with each other. A correlation between mother and daughter cell generation times has sometimes been reported for bacteria,⁽³⁾ but this correlation has not been observed in our studies.⁽¹⁾

A further analysis of our published data for *Escher-*

ichia coli B/r⁽¹⁾ has been undertaken, because it is evident that a knowledge of the degree of dependence between related cells will strongly influence the kind and complexity of theoretical models for the growth of cell populations. These data were obtained from cinematographic records of the growth and division of single cells into microcolonies upon a nutrient agar surface. Generation times were determined for each cell as the period between the appearance of a septum in its immediate ancestor to the time of appearance of its own septum, dividing it into individual daughter cells. The data were obtained from four microcolonies which were photographed for periods of three to five generations.

FIG. 75.—The dependence of the variance of the sum of successive generation times upon the number of elapsed divisions. The vertical bars above and below each point depict its estimated error.

PROGRESS REPORT

Our data provide additional evidence for the inheritance of factors that affect cell interdivision times in progeny lines, and also evidence that interdivision times are determined by two major and competing factors. Analyses of data are presented below.

The Dependence of the Variance of the Sum of Successive Generation Times upon the Number of Elapsed Divisions

Two extreme cases are considered. First, successive divisions may be completely independent. In this case, the variance of the sum, $\text{Var } S$, of the successive generation times $T_1, T_2, \dots, T_n$ will be proportional to the number n of elapsed divisions,

$$\text{Var } S = n \text{ Var } T,$$

because the variance for any generation T is constant. This kind of relationship is shown in by line A in Figure 75.

At the other extreme, the variance will remain constant over successive divisions if fluctuations in each generation time are completely compensated during the

initial part of the next cell generation:

$$\text{Var } S = \text{Var } T.$$

Line B in Figure 75 shows this kind of constancy.

From Figure 75 it is clear that the experimental data do not agree with either extreme. Instead, the variance appears to increase rather uniformly for the first three generations, after which it remains relatively constant. These results indicate that the generation times of cells are uncorrelated with those of their more immediate ancestors. However, these generation times are not independent since $\text{Var } S$ approaches constancy after three generations. This result requires the presence of long-term compensatory processes that reach their greatest effectiveness after a span of three generations.

Correlations between Ancestral and Progeny Generation Rates

Possible correlations between a given generation, g , and any one of its ancestral generations, $g-i$, where $i = 1, 2, 3$ or 4, were tested by examining cell-generation rates (reciprocals of generation times) for their deviations from the mean generation rate for all cells. The fraction of related pairs with like deviations from the mean was compared with that expected by chance, and the significance of the result was assessed by t test. Comparisons were also made against the sums of the deviations for two or more ancestral generations. The

TABLE 55. CORRELATION BETWEEN GENERATION RATES IN ANCESTRAL LINES

Ancestral generation (s)	N	L	t	Probability, %
$g-1$	160	80	0	NS
$g-2$	144	65	1.166	NS
$g-3$	112	42	2.637	<1
$g-4$	64	36	1.008	NS
$g-1, g-2$	144	61	1.834	NS
$g-2, g-3$	112	41	2.825	<1
$g-3, g-4$	64	30	0.499	NS
$g-1, g-2, g-3$	112	41	2.825	<1
$g-2, g-3, g-4$	64	36	1.008	NS
$g-1, g-2, g-3, g-4$	64	30	0.499	NS

Deviations from the mean cell generation rate were compared for each cell of an arbitrary generation g and its ancestors in earlier generations $g-i$, where $i = 1, 2, 3$, or 4. A comparison was also made with the average rate for sums of ancestral generations. In the table, N is the number of pairs of values, and L is the number of pairs with deviations of same sign. These values were compared by t test. The probability of chance occurrence is given in the final column; NS indicates that the difference was not significant.

FIG. 76.—Distributions of relative cell lengths at birth and at division. Lengths of each cell at birth and at division are connected with a vertical line. The two curved lines are approximations drawn as aids to visualization of the distributions.

results, (Table 55), show a significant, negative correlation between the generation time of any cell and that of its great-grandmother ($g-3$), but not with the generation time of any other single ancestral generation. Furthermore, this correlation is confirmed by the negative correlation between the progeny generation, g , and the average value for generations $g-2$ and $g-3$, as well as the similar correlation with the average value for all three preceding generations.

Thus it is apparent that although correlation of the generation times of mother and daughter cells is weak or absent, a compensatory factor is carried over into the daughter cells; after two further generations this factor accounts for a negative correlation of such magnitude that it is not obscured by averaging the rates for this generation with those of the two preceding generations.

These correlations are in complete agreement with the variance analysis.

Dependence of Generation Time upon Cell Size

In an attempt to relate generation time to cell size, the relative lengths of cells at "birth" and "death" were measured on the original photographs of one microcolony. The results are presented in Figure 76. From this figure it is clear that the length of a cell at

birth does not determine its generation rate. Instead, for most birth lengths there are two different average values of generation rate, and correspondingly, the cells in the two classes grow to different final lengths. Also, the two classes show more extreme differences in growth rate as the birth length of the cell is increased.

This dichotomy indicates that the generation time of a cell is under the control of at least two major, and opposing factors. Thus, the time of cell division is neither set by the birth size of the cell alone nor by the terminal length of the cell at division.

From Figure 76 it can also be seen that the increase in cell length between divisions depends upon the generation time; greater increases tend to occur in cells with longer generation times.

CONCLUSIONS

Most mathematical models of the statistics of the cell division process (the age distribution of cells) and the growth of cell populations have assumed that generation times are independent, except for a possible sister-sister correlation. Our results refute the generality of this assumption, and show instead that the ancestral dependence of generation time in our cultures is far greater than had been anticipated previously.

Furthermore, generation times were not determined

by a critical birth length or final cell size at division. For each birth length (except perhaps for extremely large cells) generation time and cell length at division fall into one of two classes. Thus, there are at least two major factors, related to cell size at birth and operating in opposing directions, in the control of generation time.

REPLICATION OF DNA

Herbert E. Kubitschek and T. R. Henderson*

We propose a model for the replication of DNA. We assume that replication occurs through the incorporation of hydrogen-bonded base-paired deoxyribonucleoside triphosphates, bonded together according to the structural code, A with T and G with C. Pairing will occur between a replicating, parental base pair and an incoming base pair if the latter satisfies a replication code. The replication code pairs A:T with T:A and G:C with C:G. This code for each base pair is established by the atoms attached to the 5 and 6 positions of the pyrimidine and the 6 and 7 positions of the purine. When replication codes match, the *complementary* pairs can approach each other closely enough within, and at the end of, the large groove of the parental duplex to trigger the action of the replicating enzyme.

In order that both incoming triphosphates can be attached to daughter strands, we assume that the replication enzyme is an allosteric oligomer, possibly a dimer or a tetramer of the well-known DNA polymerase of Kornberg. When this allosteric enzyme is triggered, the 5'-triphosphates of the incoming pair are attached, one to each newly-forming daughter

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strand, thereby extending the 3' to 5' backbone of one daughter strand and the 5' to 3' backbone of the other. Pyrophosphate is released with each attachment. Furthermore, with these reactions the hydrogen bonds of the complementary pairs are broken, and the single bases are now free to form structural hydrogen bonds with their complementary bases in the daughter duplexes. The hydrogen bonds of the incoming pair are broken because of the configuration of the daughter strands. It is assumed that the newly-forming backbones are in rather close juxtaposition at the replication fork as shown in Figure 77.

Predictions of the replication code are in agreement with experimental results for mutagenesis with 5-bromouracil and with nitrous acid. In addition, the replication code would provide for a much smaller error rate in the incorporation of bases than does a structural code alone. Finally, it should be pointed out that our model is consistent with the linear synthesis of DNA by a single allosteric enzyme, and that it permits an explanation for the absence of segregation from newly-formed mutants induced by some mutagens; a master strand specifies the coding sequence in newly-forming DNA when base pairs are mismatched, as in Figure 77.

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FIG. 77.—Courtauld model of the proposed configuration for replicating DNA. The parental helical duplex at the top is replicated to two daughter duplexes below. Replication occurs at the fork, in the replication region, which in this figure is the empty region with a rather triangular shape. The replication region is defined by the parental base pair awaiting replication, at the bottom of the parental duplex (with the bases indicated by 1 and 2), and by the daughter base pairs replicated last and therefore at the top of each daughter duplex (3,4 and 5,6). The parental base pairs were mismatched for this figure, with adenine, A(1) paired against cytosine, C(2), in this order in the large groove of the parental helix. The bases in the large groove of the right-hand daughter helix are also visible. The phosphodiester backbones lie at the edges of each duplex. These backbones are visible as the chains of oxygen atoms (large, dark grey hemispheres, some with negative signs upon them) interspersed with hydrogen atoms (small, white hemispheres). For greater clarity, the 5'-triphosphate that terminates the growing end of the daughter duplex at the right (7) was allowed to fall to a position below the uppermost, right-hand daughter base pair to which it is attached.

PHOTODYNAMIC MUTATION IN CONTINUOUS CULTURES

Robert B. Webb and Herbert E. Kubitschek

POSE AND METHODS

Acridine dyes and other similar compounds are known to bind to DNA *in vitro*. These compounds have been associated with a wide range of biological effects

including mutation in phage,⁽¹⁾ dark antimutagenesis in bacteria⁽²⁾ and yeast,⁽³⁾ and photodynamic mutagenesis in bacteria.⁽²⁾ Some of these dyes have been shown to bind to DNA by intercalation, whereby the

FIG. 78.—Photodynamic mutation rates (T5 resistance) with *E. coli* B/r/1, try⁻ in the presence of acridine orange, proflavine or 5-aminoacridine at different rates of growth.

flat three-ring molecule is inserted between adjacent base pairs in the double helix.⁽⁴⁾ Furthermore, methylene blue-sensitized photodynamic action on DNA has been shown to affect guanine preferentially, leaving the other bases and the phosphate-deoxyribose chain largely unaffected. This preferential action occurs in cells, in free DNA and in mixtures of the four bases.⁽⁵⁾

The purposes of this study are 1) identification of the structural and physical requirements for dye-sensitized mutation, 2) identification of the mode of binding associated with mutagenesis, and 3) general elucidation of basic mechanisms of mutagenesis, DNA replication, and mutation expression.

Techniques used have been presented in detail elsewhere.^(2, 6) Continuous cultures (chemostat) of *E. coli* B/r/1, try⁻ or *E. coli* WU36 (tyrosine⁻) were grown in a minimal medium containing 1) excess tryptophan and a limited supply of glucose or 2) excess glucose and a limited supply of tryptophan. Mutation to resistance to the bacteriophage T5 was assayed by plating on nutrient agar containing iron in the presence of excess phage. Resistant colonies and total counts were made after 24 hours incubation at 37°C. Dyes were added in concentrations of 5×10^{-7} M to 10^{-5} M. For dark experiments the chemostats were placed within a light shield and, in most cases, also in a darkened room. Light was supplied by a 4-watt, white fluorescent lamp placed 3 to 25 cm from the growth tube. Light intensities, as measured with a Westinghouse illumination meter, ranged from 830 foot-candles to 75 foot-candles.

Thermopile measurements with this light source indicated that 1 foot candle was equivalent to 0.7 ergs/mm²/sec.

PROGRESS REPORT

Several acridine dyes of varying complexity have been studied. These include acridine yellow (2,8-diamino-3,7-dimethylacridine), acridine orange (2,8-bis-dimethylaminoacridine), proflavine (2,8-diaminoacridine), 5-aminoacridine, and atebrin (5-(8-diethylamino- α -methylbutyl) amino-3-methoxy-8-chloracridine). In addition, methylene blue, a dye with certain similarities to the acridines, has been studied.

For acridine orange a mutation rate of about 34 mutants/hr/ μ M/100fc/10⁹ cells was obtained over a wide range of growth rates, dye concentrations and light intensities (Figure 78). This mutation rate was independent of growth rate over the tested range of 0.11 to 0.45 divisions per hour. Tryptophan-limited cultures seemed to fall in the same distribution as those limited with glucose. The mutation rate for proflavine was about 95 mutants/hr/ μ M/100fc/10⁹ cells and was about 4 mutants/hr/ μ M/100fc/10⁹ for 5-aminoacridine (Figure 78). In all three cases, the mutation rates were roughly constant with time and independent of division rate. With acridine yellow mutation rates were at the same as with proflavine. Values with methylene blue were about the same as with 5-aminoacridine. Atebrin was only slightly mutagenic in the light, giv-

ing values considerably lower than 5-aminoacridine. Table 56 presents a compendium of these and other biological effects observed with these dyes. These interrelationships are under study.

Of these dyes only atebrin was mutagenic in the dark. Acridine orange, 5-aminoacridine and proflavine, in the dark, significantly reduced the spontaneous mutation rate. Dark results on antimutagenesis with acridine yellow and methylene blue were inconclusive (Table 56). The dark mutagenesis of atebrin is unexplained, although the additional complexity of this molecule may be the basis of this effect.

A Bunsen-Roscoe reciprocity plot of the acridine orange data is shown in Figure 79. The light intensity ranged from 75 to 520 fc. The dye concentration varied from 1 to 10 μM . The mutation rate was roughly proportional to the product of the dye concentration and light intensity. Again, the tryptophan-limited cultures fell into the same distribution as those limited with glucose.

Photodynamic induction of T5 resistance is characterized by a relatively short delay before expression (Figure 80). The mean value obtained for delay using acridine orange as the sensitizing agent, was 2 divisions. This value was independent of growth rate over the range tested. Furthermore, the delays obtained with proflavine, 5-aminoacridine and methylene blue were about the same as those obtained with acridine orange. The mean delay, for the same mutation and bacterial strain with caffeine and ultraviolet light, was previously observed to be 5 divisions (Figure 3, Reference 6). This delay was also found to be independent of growth rate. Mutation by 2-aminopurine, in this system, showed an intermediate period of delay.⁽⁷⁾

Using acridine orange, photodynamic mutation to T5 resistance and to tyrosine independence was also studied in chemostat cultures of *E. coli* WU36. Muta-

FIG. 79.—Relationship of mutation rate (T5 resistance) to the product of light intensity and acridine orange concentration.

FIG. 80.—Mean delay of expression of T5 resistance for different photodynamic mutagens, caffeine and ultraviolet light at different rates of growth. Caffeine and UV data are summarized from Kubitschek and Bendigkeit^{6, 7}.

TABLE 56. SUMMARY OF FOUR BIOLOGICAL EFFECTS (*E. coli* B/r/1, try⁻) OF FIVE ACRIDINE DYES AND METHYLENE BLUE

Dye	Biological effect*			
	PhM	PhI	DM	P UV
Acridine yellow	++++	++	0?	++
Acridine orange	++++	++++	—	+++
Proflavine	++++	++	—	++++
5-Aminoacridine	++	+	—	+
Atebrin	+	+	+	+++
Methylene blue	+	++	0?	+

PhM, Photodynamic mutagenesis; PhI, Photodynamic inactivation; DM, Dark mutagenesis; P UV, Protection against UV effects. +, positive effect; 0, no effect; —, negative effect.

tion to T5 resistance occurred at about the same rate as in *E. coli* B/r/1, try⁻. Tyrosine revertants did not increase significantly in the chemostat. However, with nongrowing cells, using doses of dye and light that killed 50 to 90% of the cells, significant numbers of tyrosine revertants have been obtained. These conflicting results have not been explained.

The mutagenic effect with acridine orange was not observed in the absence of oxygen, a result in accord with other photodynamic processes. Partially bleached acridine orange produced a low mutation rate. In a

chemostat culture exposed to acridine orange and light, mutant accumulation ceased about 2 divisions after the light was turned off. These results suggest that the mutagenic agent is not a stable photoproduct of the dye acting as a chemical mutagen. The constancy of mutation rate with respect to time over a wide range of growth rates and the proportionality of mutation rate with the product of light intensity and dye concentration strongly support the photochemical nature of events leading to the primary lesion in the DNA of the cell.

The short mean delay of expression of 2 divisions (instead of 5 divisions obtained with UV light and caffeine) suggests that the initial alteration induced by photodynamic action is much closer to the fundamental alteration in the genetic material than occurs with either UV light or caffeine. A mean delay of about 2 divisions implies that phage adsorption sites on the cell surface have a lifetime of less than 1 division, or that these sites are rapidly destroyed in resistant cells. These results are in accord with those of Kubitschek⁽⁸⁾ who observed a lack of segregational division for visible light-acridine orange mutagenesis.

Our results support a direct action on DNA as the basis of photodynamic mutagenesis; however, the nature of this lesion is not revealed. The intercalation model for binding of acridine dyes to native DNA⁽⁴⁾ provides a physical basis for the light-mutagenic effects observed. The finding that 5-aminoacridine is an effective photodynamic mutagen shows that two amino groups, such as are present on proflavine, are not an absolute requirement for induction of mutation. This result differs from the findings of others⁽⁹⁾ that two amino groups in the 2,8 positions were required for photodynamic inactivation of the bacteriophage T2. As the two amino groups should greatly facilitate ex-

ternal binding to phosphate groups across the double helix, our finding favors internal binding (including intercalation) as the physical basis for mutagenesis. In addition, these dye-light results might be expected on the basis of *in vitro* results of Freifelder *et al.*,⁽¹⁰⁾ who found that DNA was degraded in the presence of acridine orange, oxygen and light. This degradation involved both depolymerization and destabilization of secondary structure and was believed to be due to oxidation of deoxyribose or one or more of the bases.

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AN OXYGEN EFFECT IN THE PROTECTION BY ACRIDINE ORANGE AGAINST INACTIVATION AND MUTAGENESIS BY ULTRAVIOLET LIGHT

Robert B. Webb

PURPOSE AND METHODS

Fluorescence studies have demonstrated specific energy transfer between DNA and acridine dyes in DNA-dye complexes. In such complexes, quenching of normal dye fluorescence is evidence that energy absorbed by the dye can be transferred to the DNA.^(1, 2) Furthermore, the observation of a relative increase and delay in the normal fluorescence of the dye (sensitized fluorescence) suggests that energy absorbed by DNA

can be transferred to the dye.^(1, 3) Fluorescence quenching in DNA-dye complexes may be associated with photodynamic effects. Sensitized fluorescence may be the basis of protection against UV damage to bacteriophage DNA in the presence of acridine orange.⁽⁴⁾ The occurrence of sensitized fluorescence in these *in vitro* systems^(1, 3) suggested that dye molecules bound to DNA and excited through energy transfer might be capable of participating in a process comparable to

photodynamic action. Since oxygen is usually required for photodynamic effects, oxygen might modify the amount of protection by acridine orange against effects of ultraviolet light. This study was designed to test this prediction as a part of a more general investigation on dye-nucleic acid interactions in bacteria.

Escherichia coli B/r/1, try⁻ and *Escherichia coli* WP2 (tryptophan requiring) were used in these studies. Stocks were maintained on nutrient agar (Difco). Experimental cultures were incubated at 37°C on nutrient agar for 2 to 3 days. Suspensions for experiments were prepared by dispersing a loop of surface growth in 0.1 M sodium phosphate buffer. In most experiments, this was followed by a 100-fold dilution in distilled water, giving a final buffer concentration of 10⁻³ M. Survival was measured by spreading 0.1 or 0.2 ml of appropriately diluted sample on nutrient agar plates. Colonies were counted after 24 hours' incubation at 37°C.

Reversion from tryptophan requirement to tryptophan independence in *E. coli* WP2 was measured by plating on a medium containing M9 salts, 200 mg/l glucose and 0.75 mg/l tryptophan. This medium allows for phenotypic expression of the induced mutants.

Dose rates, measured with a thermopile for the dif-

ferent experimental conditions, were corrected for shielding of the dye and cells in the irradiated suspensions. Shielding factors were less than 12%.

The source of UV radiation was a low pressure mercury lamp with 90% of output at 2537 Å. The irradiation chamber was designed to provide a flowing gas over the surface of a thin suspension of cells. This stream of gas (O₂, N₂ or air) produced a stirring action on the cell suspension.

For the visible light irradiations an 18-mm test tube, with a capillary tube inserted at the bottom for aeration and stirring, was positioned between two small fluorescent lamps placed 24 mm apart. Each lamp contained two 4-watt fluorescent bulbs (Sylvania F4T5/CW).

The inactivation curves are approximately described by the function

$$S = 1 - (1 - e^{-kD})^n,$$

where S is the fraction surviving, D is the dose in ergs/mm², k is the inactivation constant, and n is the y axis intercept. k is the slope of the high dose region of the response curve, and n is an expression of the size of the shoulder of the curve. The application of this function, and the biological implications of n and k have been discussed by Powers.⁽⁵⁾

PROGRESS REPORT

The dose-inactivation response of *E. coli* B/r/1, try⁻ to 2537 Å UV is shown in Figure 81. No oxygen effect was observed under these conditions. The absence of an oxygen effect for UV inactivation has been reported previously.⁽⁶⁾ In the presence of acridine orange (AO) and oxygen, UV sensitivity was substantially reduced (Figure 82). The UV sensitivity was further reduced under anoxia. The ratio k_{O_2}/k_{N_2} was about 3.5 at two dye concentrations. The protection ratio observed at 2×10^{-5} M AO under anoxia, k_{N_2} (no dye)/ k_{N_2} (dye), was 14.

In addition to an effect on UV sensitivity, the presence of AO resulted in a significant change in curve shape (Figure 82). In the absence of AO, n was 10 to 15 for this strain under these experimental conditions. When AO was present at concentrations of 5×10^{-6} M or 2×10^{-5} M, n was reduced to about 5 in the presence of oxygen. In the absence of oxygen, n was 1.0 to 1.5 at these concentrations of AO.

If oxygen depression of AO protection against UV damage is related to the photodynamic efficiency of the dye, an acridine that is a weak photodynamic agent should not show such an oxygen effect. The acridine atebrian is a much weaker photodynamic agent than AO under these conditions (Figure 83). Also, atebrian is a very weak photodynamic mutagen for the induc-

FIG. 81.—Ultraviolet dose-inactivation responses of *E. coli* B/r/1, try⁻ in the presence and absence of oxygen.

FIG. 82.—Ultraviolet dose-inactivation of *E. coli* B/r/1, try⁻ in the absence of dye, in the presence of $5 \times 10^{-6} M$ and $2 \times 10^{-5} M$ acridine orange and air, and in N_2 .

tion of resistance to the bacteriophage T5 in continuous cultures in this strain, whereas, AO is an efficient photodynamic mutagen for T5 resistance in continuous cultures.⁽⁷⁾

Results obtained for UV inactivation in the presence and absence of oxygen at two concentrations of atebrin are shown in Figure 84. Although atebrin is an effective protective agent, there is no significant oxygen effect in the response at either concentration. Atebrin is more efficient than AO as a protective agent against UV damage in the presence of oxygen and is less efficient in its absence.

Oxygen depression of the protection of AO against UV effects is not limited to inactivation of colony forming ability. Cells of *E. coli* WP2 show both increased survival and decreased mutation from exposure to 2537 Å UV in the presence of $5 \times 10^{-6} M$ AO and air (Figure 85). AO protection for both inactivation and mutation is substantially increased under anoxic conditions. The oxygen effect is at least as large for mutagenesis as for inactivation.

The magnitude of the protective effect of these two

acridine dyes is such that for AO under anoxia at $2 \times 10^{-5} M$, more than 90% of the damage from 2537 Å ultraviolet light is prevented in the high dose range. This suggests the existence of a highly efficient mechanism for the harmless dissipation of energy absorbed by the sensitive molecules of the cell. Intercalation of dye molecules between bases within the DNA helix⁽¹⁾ could provide a physical basis for this efficiency.

The large sensitizing effect of oxygen on UV damage in cells containing AO supports the hypothesis that dye molecules bound to DNA and excited through energy transfer can participate in a photodynamic-like process. If this process does occur, it is required that the same dye molecule be capable of participating in both the protective process and the damaging action. Thus, many or all of the binding sites may be common to the two processes.

Protection against UV inactivation by acridine dye results in a reduction of both the slope k and the axis intercept n . In some cases n is reduced to 1, the complete removal of the shoulder. The shoulder on the UV inactivation curve may be the consequence of some

FIG. 83.—Visible light dose-inactivation responses of *E. coli* B/r/1, try⁻ in the presence of oxygen and atebtrin or acridine orange.

FIG. 84.—Ultraviolet dose-inactivation responses of *E. coli* B/r/1, try⁻ in the presence and absence of atebtrin and oxygen.

FIG. 85.—Ultraviolet dose-inactivation and dose-mutation responses of *E. coli* WP2 in the presence and absence of acridine orange and oxygen.

kind of repair process which is relatively more efficient at low doses than at high doses.⁽⁵⁾ Removal of the shoulder under this circumstance may be the consequence of the reduction of the repairable damage with greater efficiency than the nonrepairable damage.

A scheme for the protection against UV damage may involve the following steps:

where Step 4 is a photodynamic (-like) process which would also occur after irradiation of the DNA-AO complex with 4900 Å light. Steps 1 through 3 occur with equal efficiency with AO and atebrin, whereas step 4 is efficient with AO, but is inefficient with atebrin. The role of oxygen in photodynamic processes is still unknown, although evidence for the formation of a peroxy radical has been presented.⁽⁸⁾ It is likely that photodynamic processes involving a complex of the substrate and sensitizer differ in a fundamental way from those not involving such binding.

Although *in vitro* experiments with DNA-acridine dye complexes, in which quenching of dye fluorescence and excited fluorescence have been demonstrated, present interesting parallels to the biological experiments reported here and elsewhere, precise mechanisms re-

main elusive. The analogy is more direct for acridine protection than for photodynamic action. Energy absorbed by the DNA becomes transformed to a delocalized triplet exciton. This energy, because of a strong overlap between DNA triplet and dye singlet states, can be transferred to the dye and then be emitted as fluorescence with high probability.⁽³⁾ This mechanism fits both the *in vitro* results and the biological data.

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THE EFFECTS OF PURINE COMPOUNDS ON MUTATION: SITES OF CAFFEINE BINDING IN BACTERIA. (ESCHERICHIA COLI)

Harold Bendigkeit

PURPOSE AND METHODS

It is known that caffeine and related methyl xanthines are mutagenic for growing bacteria, while certain purine ribosides reduce both methyl xanthine-induced and spontaneous mutation rates.⁽¹⁾ Although caffeine rapidly accumulates within cells to the level of its external concentration,⁽²⁾ it is neither incorporated into nucleic acids nor altered chemically.⁽³⁾ Intracellular caffeine of cells exposed to caffeine in the absence of an energy source is completely removed by washing. However, a portion of the caffeine accumulated by metabolizing cells remains complexed within the cells. The amount of caffeine bound is dependent

upon both the concentrations of caffeine and antimutagenic purine ribosides in the external medium.⁽²⁾

In tryptophan-limited chemostat cultures, the mutation rates to bacteriophage T5 resistance and concentration of bound caffeine are proportional to the caffeine concentration in the growth medium; both mutation rate and bound caffeine are similarly depressed in the presence of antimutagenic purine ribosides. Thus, only complexed caffeine (not free caffeine) appears to induce mutations.⁽²⁾

The location of the intracellular complexes and the identification of the components involved should assist in understanding the mechanism of caffeine-induced

mutagenesis. Electrophoresis of the contents of mechanically disrupted cells has been utilized for this purpose because chemical extraction of cell constituents results in dissociation of bound caffeine.

Escherichia coli, strain B/r/1, try⁻ (tryptophan-requiring), cells were grown in glucose-salts media with C¹⁴-caffeine.⁽⁴⁾ After washing in salt solution to remove labeled medium and unbound caffeine, cells were disrupted in a French pressure cell. Cell components were separated electrophoretically on cellulose acetate strips. Protein was detected with 0.2% light green SF stain in 10% acetic acid. The movement of nucleic acids was measured by staining with azure A-SO₂, as well as by absorption and fluorescence under ultraviolet light. The migration of C¹⁴-labeled caffeine was followed by counting sections of the strips with either a proportional counting chromatogram scanner or a low background Geiger counter.

PROGRESS REPORT

Acid extraction procedures on cells containing only bound caffeine result in the liberation of caffeine along with partially hydrolyzed nucleic acids. Upon electrophoresis of such hot acid-soluble extracts, only free caffeine is detected. This caffeine has dissociated from nucleic acid, nucleoprotein or another protein constituent of the cells.⁽²⁾

Low-voltage electrophoresis, on cellulose acetate strips, has been utilized in an attempt to separate the cell constituents without dissociating the bound caffeine. The protein, DNA, and RNA components of cells disrupted by rapid decompression and shearing can be separated electrophoretically. However, no complexed caffeine is detected in the migrating material when the separation is performed in media with an acid pH. The only mobile C¹⁴-activity observed is that attributable to free caffeine.

When cell material is prepared and separated in a solution of growth-medium salts of pH 6.8, free caf-

feine moves toward the cathode. A portion of the C¹⁴-activity still migrates as free caffeine. However, approximately one-half of the migrating C¹⁴-activity is observed with the DNA component. In order of relative mobility, RNA, DNA and protein, all move toward the anode. Separation of these entities is complete enough to determine that measureable C¹⁴-caffeine activity resides only in the area that contains DNA.

If the caffeine found with DNA is that portion of bound caffeine not dissociated during cell disruption and electrophoresis, caffeine is bound either to DNA or a protein (perhaps a polymerase or nuclease) which is, itself, attached to DNA. Thus caffeine, in electrostatic association with DNA, could affect DNA replication and repair by a number of mechanisms. Alternately, the noncompetitive inhibition of caffeine binding by antimutagens suggests DNA-associated enzymes as the possible complexing sites.⁽²⁾ Present evidence does not permit a choice between these alternatives.

The uncertainties associated with determinations of caffeine-complexing sites in this study have led to an additional investigation of the role of caffeine in the inhibition of DNA-repair. Determination of the specific effects of caffeine on the removal of DNA lesions and repair synthesis is in progress.

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THE RELATIONSHIP OF DNA SYNTHESIS TO GENETIC RECOMBINATION IN *ESCHERICHIA COLI*

Robert E. Krisch and Marilyn Kvetkas

PURPOSE AND METHODS

Bacterial recombination involving Hfr donors of *Escherichia coli* K-12 involves a linear, sequential transfer of chromosomal material from the donor cell to the recipient cell.⁽¹⁾ Two models have been proposed recently to explain the detailed mechanism of this

genetic transfer and to relate it to the normal cellular replication of DNA.

In the model of Bouck and Adelberg,⁽²⁾ chromosome transfer is initiated after the completion of the normal DNA replication cycle and the concomitant breakage of the normally circular Hfr chromosome. This model

predicts that the replication of the transferred Hfr chromosome has been completed in the donor cell before transfer can occur.

The other model, proposed by Jacob, Brenner, and Cuzin,⁽³⁾ assumes that conjugation itself triggers a new round of replication of the donor chromosome; this originates at the leading end of the chromosome. In this model, the newly-replicated donor chromosome is transferred into the recipient cell as it is synthesized.

The two models predict distinctly different relationships of DNA synthesis to chromosome transfer. Chromosome transfer can proceed normally in the absence of concomitant DNA synthesis by the Hfr cell according to the model of Bouck and Adelberg. However, a limited amount of DNA synthesis is required for completion of those cycles of replication under way prior to the initiation of transfer. The model of Jacob *et al.* predicts that chromosome transfer cannot proceed in the absence of DNA synthesis and that conjugation itself must initiate a new and special round of DNA synthesis in the donor cell.

Amino acid deprivation is thought to allow the completion of only a single cycle of DNA replication in *E. coli*.⁽⁴⁾ We are studying DNA synthesis in several strains of Hfr donor cells before and during conjugation in the presence of amino acid deprivation. The results of these experiments will be correlated with measurements of the ability of Hfr cells to form recombinants under the same conditions.

PROGRESS REPORT

Preliminary results with an arginine-deficient strain of *E. coli* K-12, Hfr Hayes, indicate that both DNA synthesis by the Hfr cell and its ability to form genetic

recombinants decrease in a nearly parallel fashion following amino acid starvation prior to mating. DNA synthesis was measured by pulse labeling with tritiated thymidine in the presence of deoxyadenosine, which promotes thymidine uptake. These results are in distinct contrast to those reported for another Hfr strain. Suit *et al.*⁽⁵⁾ found that the ability of Hfr-G6 to transfer its chromosome was completely unaffected by sufficient amino acid starvation to stop DNA synthesis completely in the nonmating cell.

Our results are most compatible with the model of Jacob *et al.* but are not necessarily incompatible with the results of Suit *et al.* because there may be strain-specific differences among Hfr cells.

Work in progress will extend these studies to other Hfr strains and will follow DNA synthesis during as well as prior to conjugation.

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Developmental Biology

THE CONTROL OF PRIMORDIAL GERM CELL FORMATION IN AMPHIBIANS

L. Dennis Smith

PURPOSE AND METHODS

Grafting experiments in amphibians have shown that the primordial germ cells are set aside from the presumptive somatic cells very early in embryonic development, and in all probability later give rise to all of the definitive gametes.^(1, 2) Additional experiments showed that primordial germ cell nuclei from *Rana pipiens* larvae were capable of promoting normal development following transplantation into enucleated eggs.⁽³⁾ By the same test, somatic endoderm nuclei

from a similar stage of development were severely restricted in their capacity to promote normal development. These results suggested a control mechanism whereby germ cells could be set aside before the onset of differentiation of somatic cells and could be maintained as undifferentiated cells throughout embryonic development.

It has been shown that ultraviolet irradiation of the vegetal hemisphere of uncleaved frogs' eggs results in animals whose gonads are partially or completely

FIG. 86.—Upper left, dissected Stage 25 larva showing the primordial germ cells in a normal unirradiated control. Upper right and lower left, two Stage-25 larvae which developed from eggs which were irradiated with a dose of ultraviolet light sufficient to cause a reduction in the number of primordial germ cells. Lower right, Stage 25 larva which developed from an egg which was irradiated with a dose of ultraviolet sufficient to eliminate primordial germ cells.

TABLE 57. EFFECT OF ULTRAVIOLET LIGHT OF DIFFERENT WAVELENGTHS ON PRIMORDIAL GERM CELLS

Wave-length, $m\mu$	Total dose, ergs/mm ²	No. of eggs irradiated	No. of normal stage 25	Survival of P.G.C.,* mean % $\pm$ S.E.	Inact. cross section, mm ² /quanta $\times 10^{-15}$
254	3300	20	20	17 $\pm$ 3.5	2.6404
	5280	20	20	14.6 $\pm$ 3.5	
	7900	20	15	8.1 $\pm$ 2	
	10,200	20	20	3.6 $\pm$ 1.3	
278	5280	20	20	37.7 $\pm$ 4.6	1.3340
	8160	20	11	21 $\pm$ 4.8	
	10,200	20	13	15 $\pm$ 4	
230†	5280	20	20	90 $\pm$ 3.1	0.1380
302	5280	10	10	90 $\pm$ 3.1	0.0759
	10,000	20	19	87 $\pm$ 2	

* P.G.C., primordial germ cells.

† Irradiation of eggs with a wavelength of 230 $m\mu$ was completely lethal at doses above 5200 ergs/mm².

TABLE 58. EFFECT OF ULTRAVIOLET LIGHT OF DIFFERENT WAVELENGTHS ON PRIMORDIAL GERM CELLS

Wave-length, $m\mu$	Total dose, ergs/mm ²	No. of eggs irradiated	No. of normal stage 25	Survival of P.G.C., mean % $\pm$ S.E.	Inact. cross section, mm ² /quanta $\times 10^{-15}$
254	3264	20	17	10 $\pm$ 2.3	6.9045
	5304	20	20	0.6 $\pm$ 0.3	
278	3264	20	20	51 $\pm$ 3.1	2.7416
	5304	20	12	25.4 $\pm$ 9.3	
	7650	20	20	2.6 $\pm$ 1.2	
230	3264	20	18	66.6 $\pm$ 1.2	0.1886
	5304	20	2	83 $\pm$ 11	
	7650	20	3	58 $\pm$ 4.6	
302	3264	20	20	92.7 $\pm$ 3.1	0.0759
	7650	20	19	75 $\pm$ 5.1	

sterile.⁽⁴⁾ This result suggested the existence of a "germinal plasm" which controls the formation of primordial germ cells. The present study was undertaken to determine if a germinal plasm exists in *Rana pipiens* eggs and, if so, to gain information on the nature of such a material.

The observation⁽³⁾ that the primordial germ cells can be identified in living *R. pipiens* larvae has permitted a quantitative analysis of the effects of ultraviolet irradiation of eggs on the formation of primordial germ cells. Fertilized eggs were irradiated either at the vegetal or animal pole with two light

sources: a low pressure mercury resonance lamp and a grating monochromator that uses a medium pressure mercury arc lamp. The irradiated eggs were then raised to a larval stage (tadpoles with the operculum complete) and examined to determine the numbers of primordial germ cells present.

PROGRESS REPORT

Initial experiments showed that ultraviolet irradiation of the vegetal pole of eggs just beginning the first cleavage division resulted in larvae, 100% of which were completely without primordial germ cells (Figure 86). The effective dose range was from 7700 to 15,000 ergs/mm². It should be noted further that, with the exception of the highest dose, all of the irradiated eggs developed into otherwise perfectly normal larvae. Irradiation of the animal pole of eggs just beginning the first cleavage division had no appreciable effect on the formation of primordial germ cells. These results support the hypothesis that some component(s) present in the vegetal pole cytoplasm of the egg controls the formation of primordial germ cells.

Additional experiments were carried out to determine the period of sensitivity of the egg to ultraviolet-inactivation of the germ line. At a dose of 8000 ergs/mm², irradiation of the vegetal pole of eggs approximately one-half hour before the first cleavage division resulted in the absence of germ cells in 60% of the larvae which developed from the treated eggs. At a higher dose (15,000 ergs/mm²), irradiation of the vegetal pole at the same time resulted in larvae which were all without primordial germ cells. Irradiation of the vegetal pole at the beginning of the first cleavage division resulted in the absence of germ cells in every case at both doses. Following the first cleavage division, the effects of UV irradiation on germ cell formation rapidly decreased. Eggs which were irradiated at the vegetal pole at the 8-cell stage did not give rise to larvae completely lacking germ cells, and in only a few cases was even a partial reduction in the number of germ cells observed. These results show that germ cell formation can be inhibited most readily by irradiation of eggs at the time of the first cleavage division. One possible interpretation of this is that the germinal plasm lies closest to the egg surface at the time of the first cleavage, and thus is most accessible to UV irradiation.

An action-spectrum determination was carried out to gain information on the nature of the germinal plasm. Four wavelengths, 230, 254, 278, and 302 $m\mu$ were used. The mean percent survival of primordial germ cells after irradiation of the vegetal pole of eggs is shown in Tables 57 and 58. These tables represent two separate experiments. The results show clearly

FIG. 87.—% peak absorbance: DNA (solid line) equals $O.D. \lambda / O.D. 260 m\mu$, concentration $40 \mu g/ml$; bovine serum albumin (broken line) equals $O.D. \lambda / O.D. 278 m\mu$, concentration $0.66 mg/ml$. Relative efficiency equals $\sigma\lambda/\sigma 254$, expressed as $mm^2/quanta$; ▲, results from Table 57; △, results from Table 58.

that a wavelength of $254 m\mu$ was most effective in inactivating the germinal plasm. Figure 87 represents a plot of the relative efficiency of the four wavelengths superimposed on a plot of the absorption spectrum for DNA and bovine serum albumin. The results are consistent with the hypothesis that the cytoplasmic material that controls germ cell formation is nucleic acid. The possible participation of protein cannot, however, be completely ruled out.

Finally, experiments were conducted to determine if the germinal plasm could be transferred from unirradiated eggs into those in which the plasm had been destroyed by irradiation. In the case of successful transfers, one might expect a portion of the injected eggs to develop into animals possessing germ cells, while all of the uninjected eggs would develop into animals without germ cells. The results of two experiments were as follows: When vegetal pole cytoplasm was transferred from unirradiated eggs into the vegetal pole of irradiated eggs, at the 2-cell stage, 23% of the animals were found to possess primordial germ cells. When the transfers were made at the 4- to 8-cell stage, 47% of the injected eggs developed into animals with primordial germ cells. Control injections of animal pole cytoplasm into the vegetal pole of irradiated eggs did not result in any animals with primordial germ

cells, and all of the irradiated but uninjected eggs developed into animals without germ cells.

CONCLUSIONS

Taken together, the experiments described above leave little doubt that a cytoplasmic component, probably nucleic acid, which is present in the vegetal pole even before the first cleavage division, acts to control the later formation of a single cell type—the primordial germ cells. Future experiments will thus be concerned with the elucidation of the exact nature of the germinal plasm.

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THE BIOCHEMISTRY OF AMPHIBIAN DEVELOPMENT

Richard E. Ecker and L. Dennis Smith

PURPOSE AND METHODS

These investigations were initiated to study the control of protein synthesis in the early (cleavage) stages of amphibian development. Tritiated leucine of high specific activity was microinjected into eggs or early embryos of the leopard frog, *Rana pipiens*, by the use of a micromanipulator. After a period of incorporation, saline soluble proteins were extracted from the eggs or embryos, precipitated with perchloric acid, dissolved in hydroxide of hyamine and then in scintillation fluid and counted in a liquid scintillation spectrometer. Amino acid analysis of the soluble pools of early embryos was made for us by Dr. A. B. Edmundson.

PROGRESS REPORT

Using this method, significant synthesis of saline soluble protein can be shown to occur in the first 6 hours after fertilization. In addition, either artificially-activated eggs or eggs which have been artificially activated and then mechanically enucleated, synthesize as much protein in the same period as do their fertilized counterparts.⁽¹⁾

If eggs are activated by injection of the leucine, the kinetics of protein synthesis can be studied by measuring incorporation after varying periods of exposure to the isotope. The increase in incorporated isotope in relation to time after injection follows the hyperbolic arm of an exponential; that is, the data fit the general equation

$$P^* = A(1 - e^{-Bt}), \quad (1)$$

where P^* is the amount of isotope incorporated into protein, t is time after injection and A and B are constants. By making certain assumptions concerning the mechanisms involved, the kinetics of incorporation indicated in Equation (1) can be predicted. The assumptions are: 1) A steady-state amino acid pool exists in the embryo, the size of which remains constant. The loss from the pool which occurs during the synthesis of new protein is exactly compensated by the breakdown of storage protein in the yolk. 2) The rate of

protein synthesis remains constant, that is, the rate of flow of leucine through the pool does not appreciably change during the early stages of development. 3) The amount of leucine injected does not appreciably alter the size of the leucine pool.

The first and the last points are easily verified experimentally. Amino acid analysis shows that each embryo contains a constant 0.012 μg of leucine through the 16-cell stage. About 18 millimicroliters of tritiated leucine at a concentration of 33 $\mu\text{g}/\text{ml}$ were injected, so the injection increased the size of the leucine pool by 3% or less.

The useful form of Equation (1) that results from the assumptions above is:

$$P^* = kL_0(1 - e^{-t/V}), \quad (2)$$

where L_0 is the amount of tritiated leucine injected; k is the proportion of the injected leucine that goes into the protein measured; f is the rate of flow of leucine through the pool; and V is the size of the pool. Since the rate of flow through the pool is equivalent to the rate of protein synthesis, Equation (2) can be fitted to the experimental data and the parameter, f , can then be evaluated to provide the rate of protein synthesis. In addition, the constant, k , can be evaluated to assess the relative emphasis of the saline soluble class of proteins in the total process of protein synthesis during these early stages of development.

CONCLUSIONS

The rate of protein synthesis in the first 6 hours of *Rana pipiens* development is about 2×10^{-3} $\mu\text{g}/\text{embryo}/\text{min}$. The rate is essentially the same in unfertilized, artificially-activated eggs. During this period, saline soluble proteins experience an increased emphasis in the total pattern of protein synthesis.

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LENS DIFFERENTIATION IN THE EARLY CHICK EMBRYO

Arthur K. Katoh and Elizabeth Evans*

PURPOSE AND METHODS

This report deals with the *in vitro* differentiation of the lens in the early chick embryo. Optic vesicles from Stage 10 (10-somite) embryos represent undifferentiated lens primordia. After 96 hours in culture, lens differentiation has occurred, based on histological and immunochemical criteria. The *in vitro* differentiation of the lens provides a suitable experimental system through which to ask questions about protein formation mediated by messenger RNA. This system was exposed to actinomycin D, a potent inhibitor of DNA-dependent RNA synthesis, to determine if the messenger RNA for the chick embryo lens is synthesized during the 96-hour culture period.

Adult, female, Dutch Belt rabbits were used for the preparation of antisera. Lenses were obtained from adult chickens, and a 25% (w/v) extract was made in Howard saline. The lens extract was then mixed with Freund's adjuvant, and injection schedules were followed according to Maisel and Langman.⁽¹⁾ Two rabbits served as unimmunized controls.

The lens primordia were cultured in organ culture dishes (Falcon Plastics). The culture medium consisted of equal parts of Hank's balanced salt solution (BSS), chicken serum, and 20% chick embryo (11-day) extract. Penicillin and streptomycin were added at 50 units per ml.

At the end of the incubation period in 1.0 μ Ci of H^3 -uridine, the lens cultures were rinsed twice in Hank's BSS and homogenized in cold 5% trichloroacetic acid (TCA) containing 100 μ g/ml of unlabeled uridine. The precipitates were collected on membrane filters (0.45- μ porosity) which had been soaked in unlabeled uridine-TCA. After drying, the filters were counted in a Packard Tri-Carb liquid scintillation spectrometer with the use of the scintillation fluid of Bruno and Christian.⁽²⁾

For histological examination, the tissues were fixed in Carnoy's fluid, embedded in paraffin, sectioned at 5 μ , and stained with hematoxylin and eosin.

Fertile hen's eggs were from white leghorn stock. Stages of the desired embryos were determined from Hamburger and Hamilton stages,⁽³⁾ and the somite number was counted for every embryo used in the experiments.

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TABLE 59. RESULTS OF EXPOSURE OF STAGE 10 OPTIC VESICLES TO ACTINOMYCIN D

Concentration of actinomycin D, μ g/ml	Period of exposure to actinomycin D	Total culture period, hr	Number of cases	Differentiation of explant
0 (control)	0 (control)	96	00	Lens differentiation in 35; neural tube in others
1.0	0-24 hr	48-72	29	Nonviable; necrotic, disintegrating explants
0.5	0-24 hr	48-72	16	Nonviable
0.5	0-6 hr	24	8	Nonviable
0.5	60-min pre-treatment	48	36	Nonviable
0.25	60-min pre-treatment	48	40	Nonviable
0.1	0-24 hr	48-72	10	Nonviable
0.1	0-6 hr	24-96	12	Lens differentiation in 2; neural tube in others
0.05	30-min pre-treatment	96	18	Lens differentiation in 16

PROGRESS REPORT

Before we began the study, it was necessary to show the absence of lens proteins in the 10-somite chick embryo. One hundred optic vesicles were homogenized, centrifuged at 5,000 $\times g$ for 15 minutes, and the supernatant was layered over lens antiserum. No precipitin reaction occurred.

After 96 hours *in vitro*, morphological differentiation of the optic cup and lens was evident in the cultures. When 50 explants were homogenized and prepared for a precipitin test, a positive test occurred.

These results indicated that morphological and chemical differentiation had taken place during the 96-hour period *in vitro*. The possibility that messenger RNA for lens proteins is synthesized during the culture period was investigated by using actinomycin D in conjunction with H^3 -uridine incorporation into 5% TCA-precipitable material.

Table 59 presents the data obtained on the effects of different levels of actinomycin D administered to Stage 10 explants. When 1.0-0.5 μ g/ml of actinomycin D was used for 6-24 hours, then transferred to normal

nutrient medium, the explants were not viable. Necrosis was also observed when the concentration was reduced to 0.1 $\mu\text{g/ml}$ for 24 hours. Consequently, our efforts were directed at determining a suitable level of actinomycin D concentration in which the explants are viable for the standard culture period. The actinomycin concentration was reduced further to 0.05 $\mu\text{g/ml}$, and cultures were treated for 30 minutes at different periods. In these experiments, lens differentiation was observed in a high percentage of cases. When actinomycin D was administered as a 30-minute pretreatment, 16 of 18 explants showed lens differentiation after 96 hours. In addition, 60 explants treated in the same way were harvested after 96 hours, homogenized, centrifuged, and the supernatant challenged with lens antiserum. A positive precipitin reaction was recorded in duplicate tests. Control sera from unimmunized rabbits were negative.

These findings raised the question as to whether the 30-minute pretreatment with actinomycin D at 0.05 $\mu\text{g/ml}$ could block RNA synthesis in the optic vesicle cultures. H^3 -uridine incorporation in actinomycin-treated cultures was suppressed by approximately 70% (50–150 cpm) below the controls (190–500 cpm) during the first 48 hours. Thereafter, the inhibition was almost complete (30–15 cpm).

CONCLUSIONS

The results reported here show that actinomycin D at a concentration that causes a 70% inhibition of RNA synthesis in the optic vesicle of the Stage 10 chick embryo does not prevent lens differentiation or the synthesis of lens proteins. If messenger RNA is necessary for lens protein synthesis, it appears that the mes-

senger either continues to be synthesized in the presence of actinomycin D, or that it is present in the optic vesicle prior to treatment with this antibiotic at Stage 10, and remains stable in its presence.

Although RNA synthesis was not completely turned off with actinomycin D in these experiments, it is difficult to conceive that enough "message" was formed to allow for the differentiation of lens and lens proteins in the cultures. Although we have no information on the kinds of RNA's being synthesized in our cultures, the work of Merits⁽⁴⁾ has indicated that actinomycin D inhibits the synthesis of all forms of RNA in rat liver.

An alternative possibility is that the system is insensitive to the antibiotic at this stage, i.e., that messenger is formed at some time before the Stage 10 embryo. The question then arises as to when, prior to Stage 10, the messenger might be formed. This problem was not within the scope of this study, but will be receiving attention in future studies of lens primordia of progressively earlier stages, which should provide information pertinent to the origin of lens messenger RNA in the chick embryo.

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A MOLECULAR MODEL FOR THE CIRCADIAN CLOCK

Charles F. Ehret and Ernesto Trucco

PURPOSE AND METHODS

It is generally believed that an endogenous time-keeping mechanism, i.e., an internal clock, is present in living systems. The aim of this project is to set up a plausible model of the circadian clock at the molecular level. Subsequently, it is planned to test the model, both experimentally and by performing approximate calculations when this is feasible.

PROGRESS REPORT

Although a great deal of work has been done by several investigators, no really convincing explanation for the functioning of the biological clock has been

presented.^(1, 2) Recently, however, it was shown experimentally that the circadian rhythms are strongly affected (and, in fact, even suppressed) by actinomycin D and by other antibiotics that influence the formation and the mode of action of messenger RNA.⁽³⁾ This suggests that the ordered sequence of events occurring in the end-to-end transcription of DNA strands into messenger RNA may act as an escapement for the biological oscillator. Such a view is consistent with the hypothesis (derived from other considerations) that the central clock must be closely linked with a basic and universal cellular process.

We shall call each DNA strand a polycistron com-

plex (pcc) to emphasize the fact that a cistron is considered the basic unit involved in each step of the transcription process. It is assumed that a step can occur only after the necessary precursor catalysts have been produced in sufficient quantity during the preceding step, and have reached their sites of action by diffusing into the nucleus from the cytoplasm, where they are synthesized. This is the model in its simplest form. The diffusion process is invoked to account for the fact that the *period* of biological clocks is known to be almost independent of temperature.^(1, 4)

One consequence of the proposed mechanism is that messenger RNA produced at one particular time of the daily cycle (e.g., 6 A.M.) should be different from messenger RNA synthesized at other times. Experiments are being planned to find out if this is so, through the technique of DNA-RNA hybrid formation.^(5, 6)

The quantitative features of the model (such as the

lengths of pcc's or of the diffusion paths involved) will be estimated, and computer simulation will be used to verify that the estimated magnitudes are reasonable.

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EPIDEMIOLOGICAL STUDIES IN HUMAN POPULATIONS— COLORADO BIRTH WEIGHT AND DEATH RATE STUDY

Harry Auerbach and Douglas Grahn

PURPOSE AND METHODS

The purpose and general methods of this study have been given in Grahn and Kratchman,⁽¹⁾ Grahn,⁽²⁾ and Grahn and Auerbach.⁽³⁾

PROGRESS REPORT

The initial phase of this study, based on published vital statistics and uranium ore reserves, was completed and published in December, 1963. Analysis of the county punch-card data for the state of Colorado was carried out since this state possesses populations residing in a wide range of altitudes (3000–11,000 feet). This analysis corroborated the initial study finding of an increased neonatal death rate with an increase in altitude of the maternal residence.

The 180,000 birth-certificate data cards for the state of Colorado for the years 1959–1962 were programmed on the IBM 704 computer to analyze the relationship of the average birth weight to the following variables: altitude of maternal residence, sex of the infant, maternal age, and length of pregnancy. These computations were completed and the following results were obtained:

1. Male infants at a comparable altitude, maternal age and length of pregnancy are heavier than female infants.
2. In the last 7 weeks of gestation the average weight gain for male infants is 152 grams/week, and for female infants the gain is 142 grams/week.
3. Within the usual limits of maternal age in pregnancy, (20–45 yrs) each year of increase in maternal age at pregnancy increases the average weight of infants by about 6 grams.
4. In Colorado, the average birth weight of male infants decreases by 42 grams per 1000 feet increase in altitude of maternal residence; female infants show a comparable decrease of 41 grams per 1000 feet increase in altitude. This appears to be a straight line decrease from 3000 feet to 10,000 feet of altitude. The equations for average birth weight of white single births in Colorado (1959–1962) are:

$$\text{Mean weight (Male)} = -2673.0 - 0.0425 A + 5.726$$

$$MA + 151.94 LP$$

and

$$\text{Mean weight (Female)} = -2467.6 - 0.0405 A + 5.878$$

$$MA + 143.1 LP,$$

where A = Altitude of maternal residence (in feet), MA = Maternal age (in years), and LP = Length of pregnancy (in weeks).

In the analysis of the Colorado data, a slight decrease in average length of pregnancy was found as the altitude of maternal residence increased. This was not very large and between the lowest altitude counties (3000–4000 ft) and those of highest altitude (over 9000 ft) the difference amounted to three-tenths of a week decrease in the average length of pregnancy. A similar slight decrease in average maternal age was found between the lowest altitude counties and highest counties. This amounted to slightly less than 1 year of average maternal age. Both of these two decreases could not account for the differences in average birth weight of about 370 grams for male and female infants born at low altitudes compared to high altitudes. The average birth weights of infants born at 3000–4000 feet was 3356 grams for males and 3236 grams for females, while the average weights at the above 9000-ft altitudes was 2995 grams for males and 2852 grams for females. Frequency distributions of birth weights by sex, maternal age and length of pregnancy for each altitude-group are being prepared to show the shift in birth weights with increasing altitude of maternal residence.

The analysis of infant-mortality rates was based on smaller sets of data and larger altitude groupings were necessary. Infants born at altitudes under 6000 feet were subject to mortality rates which were 25%–30% lower than the mortality rates among infants born at higher altitudes. These differences occurred in both sexes, prevailed when specific causes of death were compared, and were present in every period of the first year of life in which death occurred.

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MECHANISMS OF CARCINOGENESIS AND NEOPLASTIC GROWTH

MECHANISMS OF CARCINOGENESIS

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PURPOSE AND METHODS

The focus of interest in these studies is the induction of tumors by local irradiation. There is no very extensive quantitative information relating tumor formation to such parameters as dose, dose rate, and volume of tissue, or about the interplay between radiation and other conditions and agents, except in the special instance of skeletal irradiation. As a result, the assessment of the likelihood of human damage from accidental or environmental exposures resulting in localized irradiation rests on an insecure basis. The present experimental program is devoted largely to studies of carcinogenesis in skin and subjacent tissues.

1. Equipment has been developed during the year which will make it possible to deliver external beta irradiation uniformly to large numbers of mice. The exposure unit is a bank of cylindrical containers lined with polyurethane sheets containing radioactive microspheres of $\text{Sr}^{90}\text{-Y}^{90}$ or Tl^{204} . Mice are confined in plastic tubes 30 mm in diameter, fitted with a glass shield in the rounded end to protect the head and neck. The tubes holding the mice are approximately 60 mg/cm² in wall thickness. The sources in use at present are 35 mg/cm² in thickness and contain 100 μCi $\text{Sr}^{90}\text{-Y}^{90}$, or 500 μCi Tl^{204} per cm². The surface dose rate to the mouse is estimated to be 450 rads/hour from the $\text{Sr}^{90}\text{-Y}^{90}$ sources; dosimetry of the Tl^{204} sources is still in progress. Dosimetry has been done with silver phosphate glass fluorods calibrated for use with the beta emitters by J. Kastner, Radiological Physics Division,⁽¹⁾ which were embedded in Lucite phantoms inserted in the position to be occupied by animals. Theoretical surface and depth dose estimates⁽²⁾ have been closely confirmed in the case of $\text{Sr}^{90}\text{-Y}^{90}$, and dose measurements of the softer Tl^{204} are now in progress. A few test exposures have been given to hairless mice to determine tolerance to confinement and effects of prolonged exposure.

Experiments using this equipment will investigate tumor response as a function of dose, dose rate, genetic constitution, and combined treatment with initiating and promoting agents.

2. The experiment using plastic films in conjunction with 400 R x-radiation, described in the previous report, has been completed; a preliminary summary of the results is given below.

The mechanisms of sarcoma development following implantation of plastics is at present a subject of considerable debate.⁽³⁾ It appears to be dependent on the porosity of the implants and on the intactness of a large surface of material. Such findings, and the fact that a great variety of materials are active, suggest that the action is not that of a chemical carcinogen, but may be attributed to a disturbance in relationships between normally adjacent structures. On the other hand, certain materials appear to be inert and others vary in the intensity of response, leading to the suggestion that the molecular configuration of the surface may be of primary importance. On the basis of results of the first series of experiments, and of uncertainties as to the mechanism of the effect, we are now proceeding with a second series in which some chemically inert implants will be used (e.g. silastic, Teflon and graphite), and a series of millipore filters of graduated pore sizes (0.1 to 8.0 micra) will be tested. Fiberglass pads impregnated with powders of plastic agents which are active as extended sheets will also be used, and methods have been devised to insure immobilization of implants within their tissue capsules. A pilot experiment to study sarcoma production by intraperitoneally implanted plastic films is also in progress. We will also test discs of plastics, active in small rodents, when implanted in dogs, since large animals have not been employed in experiments of this sort.

3. Mammary adenocarcinoma production in Sprague-Dawley rats by external x-irradiation (400 R) is being examined as a function of age at the time of irradiation. The possible induction of malignant tumors within the benign neoplasms which occur naturally during the second year of life is also under investigation.

4. The possible relationship of continuous irradiation to tumor formation in capsular connective tissue

is being tested by the use of filter-paper discs impregnated with $\text{Sr}^{90}\text{-Y}^{90}$, laminated in Mylar sheets to prevent absorption of the radionuclides. In these experiments, castrated Sprague-Dawley female rats are being employed. Development of sarcoma around radioactive films will be compared with that around control films. Serial sacrifices are being made in order to follow responses in the cells of the surrounding foreign-body tissue capsules as well as in skin and other more distantly exposed areas.

5. Experiments with locally injected Pu^{239} have been in progress for two years. In Sprague-Dawley female rats, each rat received 8 simultaneous subcutaneous injections totalling 9.9, 2.2, or 1.3 μg of polymeric, particulate Pu^{239} . Also, because of the known high incidence of mammary tumors in intact females and of an apparent increased incidence in plutonium-injected rats, the two higher dose levels were administered to castrated females. A total of 550 rats, including controls, have been treated according to this plan. An additional group received a single injection of 1.3 μg intramuscularly into the right thigh muscles. In mice, a single subcutaneous injection of 1.3 μg of polymeric, particulate Pu^{239} has been given and its effect is being compared with that on a group receiving 1.3 μg ionic (soluble) Pu^{239} . Local and skeletal burdens have been measured as a function of time, and tumors are being examined histologically.

PROGRESS REPORT

1. Mice have been confined overnight (16 hours) in the beta-ray exposure chambers and appeared in good condition at the end of that period. Two hairless mice exposed to $\text{Sr}^{90}\text{-Y}^{90}$ at 450 rads/hour for this period (total surface dose 7200 rads) developed a thick scaling epidermite and died on the 20th and 21st days after exposure. One of these mice, sacrificed when moribund, showed essentially normal hematologic findings except for hemoconcentration (hematocrit 58%). Although portions of the intestine and of the bone marrow received doses in the thousands of rads it is believed that death was primarily due to plasma loss in the burned area. Mice receiving a 6-hour exposure and those exposed overnight to the Tl^{204} source, showed sharply demarcated erythema on the second day, but no abnormal findings have been noted in the subsequent 8 weeks.

2. All animals have died in the first experiment employing implanted plastic films, tumors have been diagnosed, and the experiment is being summarized for publication. Sections showing histologic changes in the reactive areas around implants are being held for further study.

Implants of solid sheet cellophane and polyethylene

produced sarcoma in the incidences of 19% and 13% respectively, agreeing with published figures.

In Sprague-Dawley rats, millipore implants of 0.3- μ pore size produced a 26% over-all incidence of sarcoma. Using 10- μ porous polyethylene, the normal 13% incidence associated with the solid sheet was reduced to 4%.

In the case of the two millipores it appears that 400 R x-irradiation 4 months after implantation does not affect the sarcoma incidence. Irradiation 8 months after implantation appears to reduce the sarcoma incidence by at least one-half compared to controls and to those irradiated at the earlier period. (Four sarcomas in seventy-five implants compared to 9/61 and 11/63).

3. Implanted $\text{Sr}^{90}\text{-Y}^{90}$ sources in thin Mylar film have not yet given rise to tumors. A few sacrifices have been made in order to observe tissue responses. After 2 months' exposure in the order of 1000 rads/day and more, these responses have been minimal.

4. Two years after subcutaneous injection of particulate Pu^{239} , about 35% of the injected amount remains in the area of injection. Total skeletal burdens in excess of 1 μg have appeared after the high-level injections, and bone tumors have been appearing in these rats after 360 days. Where the total injections have been below 2.6 μg , skeletal burdens are less than 0.2 μg and no bone tumors have appeared. The castrate group have shown somewhat higher skeletal burdens than the corresponding intact animals, but the significance of this is uncertain and must await a longer observation period of these animals.

Six months after intramuscular injection there is a retention of about 80% in the injected muscle.

Injection-site sarcomas in rats have begun to appear in the high-dose groups at 360 days, the same time as the beginning of appearance of bone tumors. In mice injected with particulate Pu^{239} the first bone tumor appeared at 328 days; in those injected with ionic material at the same dose (and with higher skeletal burdens) the first tumor appeared at 225 days. No tumors at the site of injection have yet appeared in mice.

When particulate plutonium is injected subcutaneously, autoradiographs show "stars" in the area of local injection indicating highly polymeric material. Single tracks are seen in the skeleton, liver, spleen, and in locally induced sarcomas indicating, as might be expected, that transfer from the site of primary deposition occurs following depolymerization or solubilization.

CONCLUSIONS

Equipment for large-scale external beta-ray exposure of mice has been developed and is in operation.

Sarcoma production by implantation of plastic films is not influenced by local exposure to 400 R x-ray 4 months after implantation, and appears to be somewhat inhibited by x-ray exposure at 8 months.

In the first two years following subcutaneous injection of particulate (insoluble) Pu^{239} in rats, it appears that the local neoplastic response is no greater than that in the skeleton resulting from material depolymerized and transported there.

BREAST DNA SYNTHESIS AND CANCER INDUCTION

Harold Sutton

PURPOSE AND METHODS

Many workers have studied the changes in sensitivity of cells to the lethal effects of x-ray correlated with the cell life cycle. It seems reasonable that an analogous relationship may exist between cell life cycles and the sensitivity to carcinogens. Two reports^(1, 2) have particular relevance to this hypothesis.

Two difficulties in studying the mechanism of carcinogenesis are: First, according to the somatic mutation theory of carcinogenesis, only the descendants of the rare and unidentified malignant cell of whole populations of normal cells are later manifest as tumor. Second, a relatively long period of time separates the initial event or events and the appearance of the tumor. During this induction period many intermediate events or influences which also play an important and distinct role in the subsequent expression of the tumor may go unrecognized.

To study this hypothesis of vulnerability of a specific phase in the life cycle of a cell, we chose to employ a target tissue with a large subpopulation of cells in the same phase of the cycle. Next, a correlation between some phase of this life cycle and tumor induction must be found. Then, it may be possible to identify an initial biochemical target or derangement leading to tumor formation in these cells.

The model system employed for testing this hypothesis has been discussed previously.^(3, 4) The target tissue is the mammary epithelium of 60- to 70-day-old Sprague-Dawley-derived albino rats. DNA synthetic and cell proliferative activities in this gland vary under the physiologically cyclic changes of the hormonal environment as reflected in the vaginal smear.^(3, 5, 6) DNA synthesis in the breast was assayed by the uptake of the DNA precursor, tritiated thymidine. The parameters of carcinogenesis are the number of tumor-positive animals, the number of multiple-tumor animals, histological differences in tumors, and

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variation in the length of the induction time. All rats were housed under standard laboratory conditions. The irradiated groups were intermixed, but the controls were housed separately from the irradiated group. The smear identity of an individual irradiated rat was not known during examinations for tumors. The light cycle was 12 hours of illumination and 12 hours of darkness.

A common population of approximately 250 rats was involved in the first experiment. Almost equal numbers of rats in diestrus-early proestrus and late estrus-early metestrus were distributed between the group to receive IV tritiated thymidine and the group to receive whole-body irradiation. The thymidine-treated group was to provide the relative predicted DNA synthetic activity values for the two smear classes receiving irradiation.

The tritiated thymidine-treated animals received one microcurie per gram body weight of 0.36 Ci/mM of tritiated thymidine via the tail vein. Two hours later they were sacrificed, and the three abdominal-inguinal pairs of mammary fat pads were dissected from each rat and frozen. Fat and water were extracted by the procedure of Tucker and Reece.⁽⁷⁾ After further drying, they were ground and dried to a constant weight. Aliquots of this dried extracted material were combusted in duplicate by the modified Schöniger combustion technique.⁽⁸⁾ The collected tritiated water was assayed for tritium by liquid scintillation techniques.

PROGRESS REPORT

In the tritiated thymidine-injected rats, as shown in Table 60, the average tritiated thymidine uptake in the late estrus-early metestrus group was over twice the uptake in the diestrus-early proestrus group. The ranges of uptakes in the two groups, however, over

Table 61 shows the preliminary tabulation of the tumors induced in the two groups of irradiated rats in the controls. Over 55% of the irradiated animals were

tumor-positive to palpation at eight months post-treatment. There appears to be no difference in the percentage of tumor-positive animals in the two irradiated groups. However, the induction time was somewhat shorter in the group of animals which were irradiated at the time of predicted higher DNA synthetic level. A few tumors with relatively high induction times occurred in the control animals. Data on multiple-tumor animals are shown in Table 62. A slightly higher incidence of animals with two or more tumors occurred in the predicted high-level DNA synthetic group. The preponderance of rats with three tumors per animal also occurred in the predicted high DNA synthesis group. Histological material regarding the distribution of benign and malignant tumors in the two treatment groups is not yet available.

A second experiment has been undertaken to measure the influence of diurnal variation on tritiated thymidine uptake by the breast of young mature rats. Echave LLanos⁽⁹⁾ showed by mitotic counts that breast epithelium in pregnant mice undergoes greater proliferative activity during the light phase of the 24-hour period than during the dark period. We chose to explore in young virgin female rats with the same vaginal smear class, differences in DNA synthetic activity which might be evident at 4-hour intervals during the 24-hour cycle. If such diurnal-associated differences occur, it would permit a test of the hypothesis under more favorable, and also physiologically normal, conditions. It may then be possible to select groups of rats for irradiation on the basis of both estrus and diurnal cycles. Such rats might be expected to have even more widely separated breast DNA synthetic activities than have been noted at present. Table 63 illustrates the differences in breast-thymidine uptake in rats in which early metestrus was observed at 4-hour intervals throughout the 24-hour period. An almost twofold difference between uptake at 4 P.M. and at midnight is observed for the animals with the same appearing metestrus smear. No data are yet available on the relation of diurnal cycle and tritiated thymidine uptake in diestrus rats.

CONCLUSIONS

Variations in the uptake of tritiated thymidine by rat breast are associated with the estrus cycle and the diurnal cycle. An identical carcinogenic dose of x-ray was given to two groups of animals with a predicted average, twofold difference in DNA synthetic activity related to the vaginal smear classification. No difference was found in the percentage of total tumor-positive rats in either group. However, there is some indication of an increase in sensitivity to x-ray carcinogenesis in animals predicted to have a higher

TABLE 60. TRITIATED THYMIDINE UPTAKE IN RAT BREAST RELATED TO THE ESTRUS CYCLE

Vaginal smear	No. of rats assayed	Average cpm/mg	Range cpm/mgm
Late estrus-early metestrus	47	375	158-784
Diestrus and early proestrus	45	159	49-515

TABLE 61. TUMOR-POSITIVE ANIMALS AND INDUCTION TIMES IN GROUPS WITH TWO DIFFERENT PHYSIOLOGIC LEVELS OF DNA BREAST SYNTHETIC ACTIVITY AT THE TIME OF 400 R WHOLE-BODY X-RAY

Predicted level of DNA synthesis	Treatment	No. of rats at risk	Rats with palpable tumors, %	Average induction time for first tumor, days
High	400 R	52	57.7	147.7
Low	400 R	55	56.4	160.3
High and Low	None (controls)	56	7.1	236

TABLE 62. RATS WITH MULTIPLE TUMORS

Predicted level of DNA synthesis	No. of rats at risk	Rats with two or more tumors, %	Rats with three tumors, %
High	52	23.1	9.6
Low	55	18.2	1.8
Controls	56	1.8	0

TABLE 63. TRITIATED THYMIDINE UPTAKE IN RAT BREAST DURING METESTRUS AS ASSOCIATED WITH THE DIURNAL CYCLE

Time	No. of rats assayed	Average cpm/mgm	Range cpm/mgm
Noon	2	453	402-505
4 P.M.	10	619	306-869
8 P.M.	12	495	251-819
12 M.N.	8	341	110-555
4 A.M.	3	380	231-529
8 A.M.	0	—	—

DNA-synthetic activity. This evidence consists of a somewhat shorter tumor-induction time and an increase in incidence of multiple tumors in the higher-level DNA synthetic activity group. Evidence has been found for diurnal effects on DNA synthetic activity in the breast of young mature virgin rats with similar vaginal smears.

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CO-CARCINOGENESIS BY CHEMICAL AND PHYSICAL AGENTS

Robert Zaldivar, Howard H. Vogel, Jr., and Francis E. Ray*

PURPOSE AND METHODS

The aims of this program are to study the mechanisms of carcinogenesis on various mammalian tissues with the combined action of chemical and physical agents and eventually of combinations of these with viruses, in terms of the multistage mechanism hypothesis.

Morris, Wagner, Ray, Stewart, and Snell⁽¹⁾ reported that 2,7-diacetylaminofluorene by the intraperitoneal as well as by the oral route of administration to rats has carcinogenic effects in various tissues including the glandular stomach, the intestines, and the liver. For the first time, the injection of a chemical carcinogen at a remote site induced gastric adenocarcinoma in the rat. The incidence of stomach and bowel cancer, when the intraperitoneal route was used, was 15% and 68%, respectively. The induction of gastric and intestinal carcinoma in mice by fast neutron, whole-body irradiation (290-580 rep) has also been reported.^(2,3) However, the criterion for induced malignancy in these reports is different from the one used by Morris *et al.* Nowell *et al.*⁽⁴⁾ found that no gastrointestinal tumor was induced in mice by 8-MeV neutrons with doses under 350 rads.

Based on this apparent threshold dose, we decided to irradiate rats with a single dose of 300-364 rads whole-body fission neutron irradiation including 15% gamma-ray contamination. The LD_{50/30} of a single whole-body exposure to fission neutrons in female rats was estimated to be approximately 380 rads.⁽⁵⁾

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TABLE 64. DESIGN OF AN EXPERIMENT TO TEST THE INTERACTION OF 2,7-FAA AND WHOLE-BODY FISSION-NEUTRON IRRADIATION ON THE GLANDULAR STOMACH, INTESTINE, AND LIVER OF 365 RATS

Group No.	No. of rats	Treatment*
I	50	None
II	40	GA†
III	40	GA + 2,7-FAA‡
IV	50	FN§
V	45	FN → GA
VI	50	FN → GA + 2,7-FAA
VII	40	GA → FN
VIII	50	GA + 2,7-FAA → FN

* Time elapsing between primary and secondary treatment was 14 days.

† Each animal treated with gum acacia (GA) receives three intraperitoneal injections of 0.5 ml of a 7% solution of gum acacia in 0.85% sodium chloride weekly for 23 weeks.

‡ Each rat treated with the carcinogen receives three intraperitoneal injections of 0.5 ml of an aqueous suspension of gum acacia containing 6 mg of 2,7-FAA weekly for 23 weeks.

§ Fission neutrons (single exposure, 300-364 rads).

PROGRESS REPORT

On June, 1965 an experiment was initiated concerning the interaction of 2,7-diacetylaminofluorene (2,7-FAA) given by the intraperitoneal route, with whole-body fission neutron irradiation on the glandular stomach, intestine, and liver in males of the Buffalo strain of rat. The design of the experiment is shown in Table 64.

CONCLUSION

Up to this moment, Groups I, II, III, IV, and V have entered into the experiment. Moribund or dead animals are being necropsied. It is too early to report trends on this project.

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DYNAMICS OF RELATIVE GROWTH

Anna Kane Laird

PURPOSE AND METHODS

In 1932, in his book, *Problems of Relative Growth*, Huxley⁽¹⁾ demonstrated the generality of a relation that had before that time been recognized for only a few isolated examples of the growth of parts of an organism. Known since as the allometric equation, this relation states that the log of the size of part A bears a linear relation to the log of the size of part B, or to the size of the whole organism. The relation frequently is given the form $y = bx^k$, where y is the size of part A, x is the size of part B, and b and k are constants. k is the slope of the log-log line in the alternate form, and $\log b$ is the intercept of the log-log line, i.e., b is an index of the size of part A when part B has unit size. Since it was assumed by Huxley that growth is fundamentally a process of self-multiplication of living substance, the allometric equation may be interpreted in biological terms as describing a constant correlation, as size increases, between the specific growth rates of the parts being compared.

A number of attempts have been made to relate the allometric equation to other growth equations that would include a time parameter for the growth of the parts of an organism. In a previous study,⁽²⁾ we have shown that the specific growth rate of an organism, or a tumor, decays exponentially over very wide ranges of growth; this fact is most economically described by an equation of the Gompertz type, which is a direct formalization of this empirical observation:

$$W(t) = W_i \text{Exp} \left[\frac{A_i}{\alpha} (1 - \text{Exp}(-\alpha t)) \right], \quad (1)$$

in which $W(t)$ is the mass of the organism or part at time t , W_i is the initial mass, and A_i and α are con-

FIG. 88.—Decay of the specific growth rate with age, head of chick embryo. Specific growth rate calculated as absolute difference in weight (ΔW), divided by absolute difference in age (Δt) for each of the original growth measurements, this absolute growth rate being then divided by the mean weight for the interval (taken at the $\frac{3}{4}$ point), and plotted against the mean age for the interval (also taken at the $\frac{3}{4}$ point). The line shown was determined by the method of least squares, and the intercept (initial rate of exponential growth of the system) is 1.29 ± 0.23 , and the slope (the exponential rate of decay of the exponential growth process) is 0.169 ± 0.017 , using natural logarithms.

TABLE 65. RELATIVE GROWTH OF HUMAN EMBRYO
(Data of Jackson, 1909)

Description	A_{112}^*	α	W_{112}^*	A_0^*	W_0^*
Whole	0.0504 ± 0.0012	0.0137 ± 0.0011	69.1 ± 7.7	0.234	0.104×10^{-3}
Regions					
Head	0.0422 ± 0.0011	0.0157 ± 0.0009	46.9 ± 3.8	0.246	0.114×10^{-3}
Trunk	0.0433 ± 0.0012	0.0149 ± 0.0010	51.0 ± 4.6	0.229	0.192×10^{-3}
Hindlimbs	0.0566 ± 0.0015	0.0144 ± 0.0010	10.4 ± 1.3	0.284	0.145×10^{-5}
Forelimbs	0.0512 ± 0.0014	0.0149 ± 0.0010	7.1 ± 0.79	0.271	0.274×10^{-5}
Organs					
Liver	0.0478 ± 0.0014	0.0132 ± 0.0012	3.92 ± 0.47	0.210	0.183×10^{-4}
Kidneys	0.0608 ± 0.0024	0.0181 ± 0.0019	0.56 ± 0.12	0.462	0.132×10^{-9}
Lungs	0.0557 ± 0.0023	0.0197 ± 0.0020	2.18 ± 0.41	0.505	0.265×10^{-9}
Heart	0.0430 ± 0.0007	0.0100 ± 0.0005	0.512 ± 0.025	0.131	0.733×10^{-4}
Spleen	0.0721 ± 0.0027	0.0105 ± 0.0014	0.033 ± 0.009	0.234	0.680×10^{-8}
Pancreas	0.0570 ± 0.0031	0.0135 ± 0.0030	0.049 ± 0.017	0.258	0.164×10^{-7}
Thymus	0.0886 ± 0.0028	0.0186 ± 0.0028	0.047 ± 0.022	0.707	0.154×10^{-15}
Adrenals	0.0485 ± 0.0016	0.0177 ± 0.0016	0.310 ± 0.041	0.351	0.114×10^{-7}
Thyroid	0.0589 ± 0.0024	0.0126 ± 0.0019	0.041 ± 0.010	0.242	0.200×10^{-7}
Brain	0.0475 ± 0.0015	0.0133 ± 0.0014	10.7 ± 1.5	0.210	0.514×10^{-4}

* Subscripts refer to days after conception at which the growth equation had these parameters. W_0 and A_0 are extrapolated values to conception. W_{112} and W_0 are weights in grams.

TABLE 66. RELATIVE GROWTH OF CHICK EMBRYO
(Data from Romanoff, 1960)

Description	A_6^*	α	W_6^*	A_0^*	W_0^*
Whole	0.856 ± 0.017	0.176 ± 0.010	0.259 ± 0.032	2.46	0.288×10^{-4}
Regions					
Head	0.567 ± 0.019	0.197 ± 0.010	0.312 ± 0.012	1.85	0.461×10^{-3}
Trunk	0.752 ± 0.055	0.134 ± 0.018	0.175 ± 0.020	1.68	0.173×10^{-3}
Neck	0.624 ± 0.066	0.098 ± 0.019	0.0269 ± 0.0035	1.13	0.150×10^{-3}
Hindlimbs	1.26 ± 0.056	0.164 ± 0.013	0.00254 ± 0.00036	3.37	0.648×10^{-8}
Forelimbs	0.818 ± 0.025	0.105 ± 0.006	0.00245 ± 0.00014	1.53	0.264×10^{-5}
Organs					
Brain	0.670 ± 0.020	0.210 ± 0.018	0.035 ± 0.005	2.37	0.108×10^{-4}
Eye	1.02 ± 0.023	0.330 ± 0.021	0.019 ± 0.003	7.40	0.764×10^{-10}
Eyeball	0.805 ± 0.027	0.227 ± 0.018	0.00022 ± 0.00002	3.15	0.727×10^{-8}
Lungs	1.09 ± 0.025	0.166 ± 0.008	0.00052 ± 0.00005	2.96	0.684×10^{-8}
Heart	0.713 ± 0.028	0.153 ± 0.013	0.0028 ± 0.00028	1.78	0.254×10^{-5}
Liver	1.05 ± 0.030	0.166 ± 0.010	0.0023 ± 0.00024	2.84	0.476×10^{-7}
Gizzard	0.962 ± 0.018	0.103 ± 0.004	0.00101 ± 0.00004	1.79	0.340×10^{-6}
Mesonephros	0.808 ± 0.020	0.298 ± 0.016	0.0018 ± 0.00008	4.83	0.243×10^{-8}
Metanephros	0.692 ± 0.023	0.113 ± 0.007	0.0011 ± 0.00006	1.36	0.294×10^{-5}
Spleen	1.08 ± 0.069	0.204 ± 0.013	0.000049 ± 0.000005	3.69	0.142×10^{-9}
Intestine	1.02 ± 0.081	0.120 ± 0.012	0.0025 ± 0.00047	2.09	0.329×10^{-6}

* Subscripts refer to days after conception at which the growth equation had these parameters. W_0 and A_0 are extrapolated values to conception. W_6 and W_0 are weights in grams.

stants. In applying this equation to the data of the present study, we have taken advantage of the fact that in the solution of the equation any time may be designated as zero time, and in this case the value for A_t and W_t will be appropriate to the time chosen; α will remain the same no matter what shift is made

in the time scale (for the derivation of the necessary transforms, see Reference 2).

PROGRESS REPORT

For the present study data were collected from the literature on the growth of the parts of the human

the mean. In contrast the values found for W_t are distributed over a range that is hundreds and thousands of times larger than the ranges exhibited by A_t and α .

The empirical estimate of the changes of the specific growth rate as the animal ages, formalized in the Gompertz growth equation, bears a meaningful relation to the allometric equation of Huxley.⁽¹⁾ For any two parts of an organism for which the values of α , the rate of decay of the specific growth rate, are nearly the same, the log of part A will necessarily bear a linear relation to the log of part B ; if the values of α are different, the allometry plot will yield a curved line.⁽⁶⁾

These two cases are illustrated in the allometry plots of Figures 89 and 90. In Figure 89, the log of the size of the intestine is plotted against that of the metanephros, and in Figure 90, the log of the size of the mesonephros is similarly plotted against the same referent. It is evident that the allometric plot of Figure 89 yields a satisfactory approximation to a straight line; for the intestine and metanephros the values for α are very similar, being respectively 0.120 and 0.113. On the other hand, when the size of the mesonephros, with a proportional rate of decay of the specific growth rate of 0.298, is plotted against that of the metanephros, with its decay rate of 0.113, we obtain a distinctly curved line (Figure 90). It is worth noting in passing that one might be tempted to fit these points with two straight lines, as indicated by the dotted line in Figure 90. However, it is clear from our analysis of the specific growth of these organs that two straight lines have no biological meaning at all, while a curved line does give an approximate description of the changing relation between the specific growth rates of these organs with age.

EFFECT OF HYDROGEN PEROXIDE-PRODUCING AGENTS ON MICE BEARING THE EHRlich ASCITES TUMOR

Robert N. Feinstein, Judith B. Howard, Joann T. Braun,
and Marilyn V. Coulon

PURPOSE AND METHODS

The theory has been advanced by Warburg⁽¹⁾ and by Holman⁽²⁾ that hydrogen peroxide, H_2O_2 , is the molecule of choice in the therapy of cancer. Although treatment of cancer-bearing animals directly with H_2O_2 would appear a priori doomed to failure because of the ubiquitous presence of catalase, there may be devices to obviate this. If, instead of injecting tumor-bearing animals with H_2O_2 per se, one injects a compound which produces H_2O_2 *in vivo*, it is possible that sufficient H_2O_2 will be released directly at the tumor

CONCLUSIONS

The specific growth rates of the parts of an organism decay proportionally with time. The constant rates of this proportional decay are usually very similar throughout the body. Since the logs of two specific growth rates will yield a straight line if, and only if, the rates of decay are the same,⁽⁶⁾ the finding of closely similar rates of decay for the parts of an organism offers an empirical basis for the nearly universal observation of the log-log relation of simple allometry.

Although most of the organs and regions of the body share a common rate of decay of the specific growth rate, there are a few clear exceptions. These necessarily give, not a straight line, but a curve on an allometric plot. The significance of this curved line lies in the fact that when the time scales of the two processes under consideration are different (α is the inverse measure of the time scales⁽²⁾), we observe one growth process slipping past the other.

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site to be therapeutic. In pursuance of this possibility, we have tested, with some success, two agents known to produce hydrogen peroxide: a) the enzyme glucose oxidase* (1.1.3.4), which oxidizes glucose to gluconic acid with the production of H_2O_2 ; and b) the compound 1-methyl-2-p-(isopropylcarbonyl)-benzyl hydrazine hydrochloride (Ro 4-6467/1),* which is known⁽³⁾ to produce H_2O_2 in solution by autoxidation.

* We wish to thank Dr. George E. Ward, of Dawe's Laboratories, Inc., Dr. W. E. Scott, of Hoffman-LaRoche, Inc., and Dr. Cameron Ainsworth, of Eli Lilly and Co., for generous gifts of glucose oxidase, Ro 4-6467/1, and 5-MT, respectively.

embryo,⁽³⁾ the chick embryo,⁽⁴⁾ and the young postnatal rat.⁽⁵⁾ For each data point the specific growth rate $[(1/W)/(\Delta W/\Delta t)]$ was determined at that age, and the succession of such specific growth rates was plotted on semilog graph paper. Figure 88 shows such a plot of the changes in specific growth rate with age, for the head of the chick embryo. The line shown was fitted by the method of least squares. The correspondence between this treatment of the data and the growth equation (1) is the following: The slope of the line of Figure 88 is a measure of the rate of exponential decay of the specific growth rate, and corresponds to α in Equation 1; the intercept of the line in Figure 88

TABLE 67. RELATIVE GROWTH OF POSTNATAL RAT
(Data of Jackson and Lowrey, 1912)

Description	A_{27}	α	W_{27}
Whole	0.107 ± 0.0193	0.0325 ± 0.0039	9.38 ± 2.62
Region			
Head	0.0666 ± 0.0031	0.0320 ± 0.0015	3.17 ± 0.09
Trunk	0.0953 ± 0.0200	0.0274 ± 0.0060	5.73 ± 1.20
Hindlimbs	0.101 ± 0.0090	0.0300 ± 0.0030	1.41 ± 0.13
Forelimbs	0.0733 ± 0.0017	0.0285 ± 0.0007	1.10 ± 0.02

* Subscripts refer to days after conception at which the growth equation had these parameters. Because α has a much lower value in postnatal than in prenatal growth, for the rat and a number of other species (unpublished results), extrapolations from the postnatal growth curve to A_0 and W_0 would have little meaning, and are omitted from this table.

FIG. 89.—Allometric plot, chick embryo, weight of intestine versus weight of metanephros, days 8-21 after conception. The rates of decay of the specific growth rates for these two organs are very similar: 0.120 for the intestine, and 0.113 for the metanephros.

FIG. 90.—Allometric plot, chick embryo, weight of mesonephros versus weight of metanephros, days 4-14 after conception. The rates of decay of the specific growth rates for these two organs are very different: 0.298 for the mesonephros, and 0.113 for the metanephros.

corresponds to A_t in Equation 1. The quantity W_t in Equation 1 is an integration constant and is not estimated by the empirical procedure.

The growth equation (1) was also fitted to the data by a least squares procedure; the weighting factor used was $1/W^2$.

In Tables 65-67 are given the values for A_t , α and W_t determined for the above sets of data. In each case, the equation was solved for an initial time taken within the data points in order to minimize the standard error on the parameters. Once the equation had been fitted, it was then possible to extrapolate the growth curve to any desired time; the values given for A_0 and W_0 in the tables are such extrapolations to the time of conception. These extrapolations are not intended to furnish descriptions of biological reality; their significance is only that in later growth, at a time when the organ or part is large enough to be weighed, it grows as though it had had the mass W_0 at the time of conception.

It is evident from inspection of the tables that the values found for A_t and α for the parts of any single organism are relatively much more similar than are the values found for W_t , either when zero time is taken to lie within the range of the data, or especially when the curve is extrapolated back to conception. With a few exceptions, most of the values for A_t and α lie within a twofold range, and for regions of the body the range is even smaller, lying within about $\pm 25\%$ of

Some similarly-based experiments were recently touched upon by Burk and Woods.⁽⁴⁾

The only tumor we have used in this study was the Ehrlich ascites tumor, carried in mice of both sexes and various ages. All injections were intraperitoneal. The glucose oxidase was dissolved in saline, with no pH adjustment. The Ro 4-6467/1 was dissolved in saline, quickly adjusted to pH 6.5-6.8, and injected within 10 minutes of initial solution. The catalase inhibitors 3-amino-1,2,4-triazole (AT) and 5-mercapto-1,2,3,4-tetrazole (5-MT),⁽⁵⁾ when used, were dissolved together with the peroxide-producing agent. Animal

weight, and survival times, were used as criteria of therapeutic effect.

PROGRESS REPORT

Glucose Oxidase

The results of eleven separate experiments with single injections of glucose oxidase are pooled in the data of Table 68. Although both sexes of animals, at a variety of ages, some treated with glucose oxidase alone and some with glucose oxidase plus AT or 5-MT, were used in these experiments, differences between experiments were so minor as not to warrant individual descriptions. In each case, however, controls were employed in which saline solution alone (or with AT or 5-MT) replaced the corresponding glucose oxidase solutions. It will be noted from Table 68 that almost half the treated animals, but less than 10% of the untreated mice, survived at least 2 weeks. A small percentage of treated mice, but no untreated ones, survived a full month. Even these 30-day survivors succumbed shortly thereafter, all of them showing solid

TABLE 68. EFFECT OF GLUCOSE OXIDASE INJECTION ON MICE BEARING EHRLICH ASCITES TUMOR

Treatment	Total No. of mice	Survivors (14 days)		Survivors (30 days)		Mean survival time, days
		No.	%	No.	%	
Saline	60	5	8	0	0	11.3
Glucose oxidase	220	104	47	10	5	13.7

FIG. 91.—Effect of Ro 4-6467/1, plus 5-mercapto-1,2,3,4-tetrazole, on weight curve of ascites tumor-bearing mice. Curve A, saline twice daily for 12 days; mean survival time 13.8 days (does not include one mouse which survived indefinitely, with no weight gain at any time). Curve B, drug combination twice daily for 5 days; mean survival time 23.6 days. Curve C, drug combination twice daily for 12 days; mean survival time 24.8 days. Crosses represent deaths of individual mice. Arrows indicate days of injection (injected morning and afternoon, at 0.01 ml/g. body weight; Ro-4-6467/1 given at 100 mg/kg; 5-MT given at 500 mg/kg).

FIG. 92.—Effect of Ro 4-6467/1 on weight curve of ascites tumor-bearing and non-tumor-bearing mice. Curve A, saline only, to tumor-bearing mice. Curve B, Ro 4-6467/1, to tumor-bearing mice. Curve C, Ro 4-6467/1, to non-tumor-bearing mice. Crosses represent deaths of individual mice. Each arrow indicates one injection. Ro 4-6467/1 was given at 100 mg/kg. Each group consists of ten mice.

tumor growths on the peritoneal wall. Mean survival time was increased by the treatment only 2.4 days (21%); there tended to be large numbers of deaths among treated animals early in the third week after tumor implantation.

Ro 4-6467/1

The Ro 4-6467/1 was injected twice daily, at 100 mg/kg each time, with no sign of cumulative toxicity. The tumor was permitted 3 days' unimpeded growth before therapy was initiated.

Figure 91 shows the effect of injecting the Ro 4-6467/1, together with the catalase inhibitor 5-MT, twice a day for 5 days, versus twice a day for 12 days, versus 12-day treatment with saline only. The delay in weight gain is obvious from the figure. The relatively small differences in survival time between the 5-day-treated group, and that treated for 12 days, warns that weight gain alone is not a sufficient criterion for measuring therapeutic success with an ascites tumor.

Figure 92 summarizes an experiment in which Ro 4-6467/1 was injected twice a day for 12 days, with no added catalase inhibitor. At the same time, a series of non-tumor-bearing mice was also injected with the drug in order to determine the effect of the drug alone on the normal growth curve. Although the drug had relatively little effect in this case on the weight gain of the ascites bearing mice, or its time of onset, the ascites weight gain of many of the treated mice was sharply reversed within a week of cessation of treatment. At 30 days, seven of the original ten mice still survived, and their average weight was very near that at the beginning of the experiment. However, six of these surviving seven mice died during the second month, all of them with evidence of the formation of solid tumors. One animal, weighing 26 grams at the start of the experiment, reached a weight of 45 grams at day 14, and was back to 26 grams at day 24. He was still alive and active at 90 days, although two very slow-growing nodules were detectable on the abdominal wall. He was then challenged with a fresh in-

jection of Ehrlich cells, but without therapy, and he succumbed in 14 days.

CONCLUSIONS

The following points follow from the above results:

a) The enzyme glucose oxidase and the compound Ro 4-6467/1, both of which may be expected to produce H_2O_2 upon injection, do affect the progress of the Ehrlich ascites tumor, as evidenced by delay in onset of weight gain and/or prolongation of survival of the host.

b) Repeated injection of Ro 4-6467/1 into non-tumor-bearing mice causes a weight loss. This effect alone, however, cannot account for the delaying effects of Ro 4-6467/1 on weight gains in ascites tumor-bearing mice.

c) Despite sometimes striking effects on weight gain and survival time, the ultimate cure rate of the Ro 4-6467/1 is negligible. A more usual effect is the increase

of survival time to a point where the animals die with multiple solid tumors but no signs of ascites.

(d) An animal "cured" of, or at least temporarily protected from, the Ehrlich ascites tumor, has not necessarily developed an immunity to the tumor.

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NEUTRON CAPTURE AND CONVERSION THERAPY WITH INTERMEDIATE-RANGE NEUTRONS

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PURPOSE AND METHODS

Successful neutron-capture therapy of tumors depends on concentration of an activatable, but nontoxic, nuclide within the lesion, followed by *in situ* activation of the nuclide by neutrons. The dose released locally should destroy the neoplasm while leaving normal tissue unharmed. Our theoretical and experimental work along these lines has been reviewed elsewhere along with prior work by other authors.⁽¹⁻⁴⁾ Previous attempts involved the localization of B^{10} coupled with irradiation by thermal neutrons. Neutron capture promoted the $B^{10}(n, \alpha)Li^7$ reaction with concomitant prompt release of 2.34 MeV of ionizing radiation in the form of an alpha particle and a Li^7 nucleus.

The failure of this system to achieve clinical cures or significant regressions^(3, 5, 6) can be ascribed, in part, to the fact that the thermal neutrons used penetrated tissue so poorly that reaction was largely confined to regions outside the tumor.^(2, 7)

Acting on theoretical considerations,^(1, 8, 9) we were able to develop the use of *epithermal* neutrons⁽²⁾ and to show that they were much more effective than thermal neutrons.^(2, 10) This has since been confirmed by others.^(7, 10) However, our theoretical studies in-

dicated further that beams of *intermediate* (i.e., 0.3-300 keV) energy neutrons would be even more effective.⁽¹²⁾ In consequence, we have directed this year's investigations toward examination of the parameters of systems involving intermediate-range neutrons.

PROGRESS REPORT

Extension of our previous theoretical studies, via transport, Monte-Carlo and narrow-beam methods,^(1, 12, 13) indicated that optimum neutron distributions for therapy would be obtained from narrow beams of neutrons in the 0.3- to 30-keV range. Although crystal monochromators may some day produce such beams at useful flux levels, they are presently unavailable.⁽¹⁾ However, neutrons in the 30- to 300-keV range are available via the $Li^7(p, n)$ reaction,⁽¹⁴⁾ and theoretical treatment and preliminary experiments⁽¹²⁾ indicated that they would be more useful than reactor epithermal neutrons on three counts.

1) They would be much less contaminated by undesirable gammas or fast neutrons.

2) They would penetrate more deeply.

3) Their availability, as closely defined beams, would greatly minimize dose to the rest of the body and, at the same time, permit use of rotation therapy.

With the latter, dose could be concentrated on the tumor while being diffused out over normal tissue.

In addition to their utility for the *localization* variety of neutron-capture therapy, intermediate-energy neutron beams provide possibilities in another direction. If an activatable nuclide could be distributed uniformly through tissue, one could enhance the possibilities of what is now called fast-neutron therapy.⁽¹⁵⁾ Since this would be accomplished by "conversion" of the low-LET fraction of fast-neutron dose to a high-LET variety, we have styled this modification *neutron-conversion therapy*.

The fundamental advantage of fast-neutron therapy lies in the fact that neutron dose is of higher LET than x or gamma dose. High-LET radiation shows little oxygen effect. Thus, the anaerobic core of many tumors is, in effect, "protected" by its lack of oxygen—by a factor of about three as compared to normal tissues. This results in a maximum effective therapeutic ratio of only 0.33 or so for such cores and would account for many of the operationally radioresistant tumors seen in conventional radiotherapy.⁽¹⁵⁾

In fact, fast neutrons do not seem to be completely free of oxygen effect. This is only what might be expected from the fact that a sizeable fraction of the total dose in tissues, from even the "purest" of fast-neutron sources, arises from photons of the H and Cl (n, γ) capture reactions. In any case, most neutron sources contribute a fair fraction of photons in their own right. All in all, the photon fraction may range from a few per cent near the irradiated tissue surface to as much as 90% for a broad 1-MeV neutron beam at 15 cm from the surface.⁽¹⁶⁾

Our concept has been the utilization of the neutron-capture effect to produce high-LET particles rather than photons. When combined with the peaked depth-dose distribution, narrow beam (for rotation therapy) and low photon contamination of $\text{Li}^7(p, n)$ neutrons, it should provide a useful and almost immediately available method for tumor radiotherapy. This is to be contrasted to the present state of localization neutron-capture therapy where problems of localization and chemical synthesis remain to be solved.^(1, 12)

We have found that mice, at least, will tolerate a lithium concentration of the order of 0.025 M, confirming previous work.⁽¹⁷⁾ If it may be assumed that Li distributes itself as uniformly in tissues as the other alkali metals, then administration of Li^6Cl , followed by narrow-beam intermediate energy neutrons, might provide a useful form of radiotherapy—one fairly free of interference by the oxygen effect. In addition, Li^6 localization in tumors has been reported⁽¹⁸⁾ and, if applicable to human tumors, would provide an additional bonus in the form of a localized neutron-capture

dose. Since the physics seems reasonable (*vide infra*) the crucial question is that of uniform distribution at the subcellular level. The range of the particles from the $\text{Li}^6(n, \alpha)\text{H}^3$ reaction, 10 and 50 μ respectively, is not so large that the considerations of microdosimetry⁽¹⁹⁾ might not be applicable. Thus, intranuclear, or at least intracellular, distribution will be important, albeit less so than for the case of B^{10} .

He^3 represents an alternative to Li^6 , but a poor one. It is hopelessly expensive at present and its solubility, even at 10 atm partial pressure, is only about 5×10^{-3} M. This would yield a specific dose only about 25% that of Li^6 at 0.02 M. Of course, uniform intranuclear distribution would be assured on chemical grounds—a necessity since the particle ranges for the $\text{He}^3(n, p)\text{H}^3$ reaction are only 18 and 0.3 μ , respectively.

From a clinical standpoint, it is probable that irradiations could seldom exceed an hour without discomfort to an already ill patient. However, it is conceivable that such irradiations could be repeated at least several times. Such dose fractionation would be advantageous from a radiobiological standpoint since recovery from photon effects is much more rapid than that from high-LET particles. Thus, fractionation would result in a smaller over-all effect of the photon fraction of the dose but a constant high-LET effect for either capture or conversion therapy.

Maximum proton-beam currents with our machines are of the order of 200 μamp (i.e., 200 μ coulomb sec^{-1}). At this rate, about 50 minutes would be needed for a tumor dose of 3000 rads with U^{235} , but about 8½ hours with B^{10} . However, machines have already been

FIG. 93.—Flux and dose distributions through phantom head irradiated with a beam of 60-KeV neutrons.

built and tested which produce 4000- μ amp proton-beam currents at the proper energy,⁽²⁰⁾ so that dose rate with intermediate-range neutrons need not be a problem in future.

Although a great many runs have now been made with $\text{Li}^7(p,n)$ neutrons, one experiment will suffice to illustrate our findings of the past year. A phantom "head", a right circular cylinder 15.3 cm in diameter and 15.3 cm high, was located with its $(r, \theta, 0)$ plane in the plane of the neutron beam. It was encased in a cube of Pb, 25 cm on a side, but located eccentrically so that the cylindrical segment closest to the beam subtended an arc of 2 cm (i.e., $0 \pm 7.5^\circ$) without Pb shielding. The head was filled with tissue-equivalent liquid⁽²¹⁾ and was provided with a 7-mm bone-equivalent "skull." Na^{23} and Au^{197} foils, U^{235} fission counters, Rossi W chambers, CO_2 -graphite chambers, Ne-Ar gamma counters or synthetic "tumors" of tissue-equivalent gel⁽²⁾ were suspended within it. A 2×2 cm, magnetically spread, proton beam at 2010 keV was caused to impinge on a 130-keV Li target deposited on Cu, as described elsewhere.⁽²²⁾ Spectrometry showed the neutrons to be nearly Gaussian in energy distribution with a peak at about 60 keV and a cutoff at 240 keV, in agreement with theory, semi-empirical computations and previous measurements.^(14, 22)

Thermal flux, total dose and gamma dose along the axis of the beam are shown in Figure 93, corrected to a "standard" tissue density of 1.04 g cm^{-3} from the liquid density of 1.12 .⁽²³⁾ Penetrations are measured from the inside of the "skull." Target to outside of skull distance was 25 mm.

Total doses at the PTR* reference point (*vide infra*) as the head was rotated through 360° , are shown in Figure 94. From these data, the doses to a 1-cm^3 tumor located at different depths have been computed, and are presented in Table 69.

Additional measurements made with other phantoms confirmed previous theoretical and experimental work⁽¹³⁾ to the effect that a 10-cm tissue block is "semiinfinite" at these energies. Thus, increasing the size of our phantom did not affect central dose distributions. Accordingly, the points at 12.5 cm can be regarded as equivalent to points 12.5 cm into a human trunk or torso, i.e., the approximate body mid-point.

In computing PTR a single reference, the total dose at its 4-cm maximum value, was taken as the dose to most sensitive nonneoplastic tissue. The rationale this was that, to date, capillaries have been the

FIG. 94.—Dose at PTR of phantom head as function of beam angle.

most critical of body tissues in neutron capture therapy.⁽³⁾ Since they are ubiquitous in tissue, the point of maximum tissue dose will also be the point of maximum dose to them. The only other candidate for most sensitive tissue along the beam line is skin, but its maximum dose is lower than that of the capillary even at 4 cm, so that choice of capillary yields a conservative figure even for skin.

Measurements of whole-body dose to various parts of a body phantom showed it to drop off exponentially with distance from the beam entrance point exactly as would be expected by extrapolation from Figures 93 and 94. Thus, tissue doses became negligible 15 cm from beam entrance, a marked improvement over the epidermal beam situation.⁽⁷⁾

CONCLUSIONS

It is evident that intermediate-energy neutrons are superior even to reactor epithermal ones. Thus the one-field PTR's for an optimized reactor epithermal facility and B^{10} at 4 and 7 cm were 1.62 and 0.97, respectively.⁽⁷⁾ This compares with 2.8 and 1.97 for intermediate-energy neutrons.

The advantage of rotation therapy is even more striking, especially with the narrow intermediate energy beam. While some rotational advantage could be obtained with a reactor epithermal beam, its radial dose distribution is not nearly as favorable⁽⁷⁾ as that

* PTR (physical therapeutic ratio), the ratio of rads delivered to the tumor to those delivered to the most critical nonneoplastic tissue in the body.

TABLE 69. CAPTURE AND CONVERSION TUMOR DOSES*

	Capture Therapy						Conversion Therapy		
	Nuclide								
	U ²³⁵			B ¹⁰			Li ⁶		
	3.5 × 10 ⁻³ 3.5 × 10 ⁻⁴			3.5 × 10 ⁻³ 3.5 × 10 ⁻⁴			2 × 10 ⁻² 2 × 10 ⁻²		
Conc. of nuclide in tumor, M/l. Conc. of nuclide in tissue, M/l.									
Penetration, cm	4	7	12.5	4	7	12.5	4	7	12.5
Nuclide dose, 1 field	2520	1750	325	252	175	32	685	472	88
Tissue dose, 1 “	100	72	18	100	72	18	100	72	18
Total dose, 1 “	2620	1822	343	352	247	50	785	544	106
Reference dose, 1 “	352	—	—	125	—	—	785	—	—
PTR, 1 “	7.4	5.2	0.97	2.8	1.97	0.40	1.0	0.70	0.14
PTR, 16 fields	15.6 (45)†			5.9 (18)†			2.10 (6)†		
Reaction Q, MeV	165			2.4			4.8		
Reaction σ, barns	582			4017			945		

* Doses are given in rads per coulomb to a 1-cm³ tumor located at the penetration given. Tissue density assumed 1.04 g cm⁻³. Reference dose taken to be that to normal tissue, at a penetration of 4 cm, in the absence of a tumor, and at the tissue concentration given. Rotation PTR for 16 fields computed from Figure 94 for 7 cm.

† Estimated by analytical methods for a tumor at 12.5 cm within a 25-cm human torso.

shown in Figure 94, so that the advantage would be relatively small.

Thus, the intermediate energy-rotation system is the most promising yet uncovered. In conjunction with U²³⁵ it is the only system which holds real promise for tumors or metastases located deep within the body.

In conclusion, the *physical* problems of capture therapy have been solved, especially since further improvement, by a factor of two or more, can be expected as the present system is refined by better collimation, higher neutron yields, higher machine currents, etc.

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THE CHEMISTRY AND PHYSICS OF NUCLIDES FOR NEUTRON-CAPTURE THERAPY

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PURPOSE AND METHODS

The failure of neutron-capture therapy utilizing B^{10} compounds and thermal neutrons can be ascribed in part to the poor tissue penetration of such neutrons, and in part to the fact that the B^{10} compounds used concentrated better in the blood than in the tumor. As a result, normal blood vessels were destroyed in preference to tumor tissue.^(1, 2)

The problem of neutron penetration has seemingly been solved by application of narrow beams of intermediate energy neutrons,⁽¹⁾ but the problems of adequate localization and dose distribution remain.⁽³⁾ Chemical and physical considerations^(3, 4) lead to the conclusion that B^{10} and U^{235} are the most promising of nuclides for capture therapy. Accordingly, we have directed this year's investigations toward an examination of those characteristics of these two nuclides most pertinent to neutron-capture therapy.

PROGRESS REPORT

Boron Chemistry

Our studies of arsenoazoprotein localization in spontaneous somatic murine tumors⁽⁵⁾ convinced us that similarly labeled borono or urano proteins should provide adequate tumor localization for therapy. We, therefore, focussed on chemical methods for the incorporation of B^{10} and U^{235} into such proteins. In this connection the elegant, recent work of Soloway, Bogoch and Burstein⁽⁶⁾ is of great interest. By almost heroic effort, they were successful in isolating proteins unique to transplanted murine gliomas, forming a rabbit antibody to these, labeling the antibody with boron and showing boron tumor/blood ratios of the order of 5-8 on subsequent I.V. injection. By using an isocyanate of $B_{10}H_{10}^{2-}$, they have been able to produce antibody proteins nearly 3 weight % in boron. With these they have achieved boron concentrations in tumor of the order of $5 \times 10^{-3} M$, a level almost certainly adequate for therapy.⁽¹⁾

Our azoprotein studies indicated that antibody formation is unnecessary and that any serum protein will localize in tumors, greatly simplifying the procedure and making a single substance applicable to all manner of tumors.⁽⁵⁾ By diazotization of $m-NH_2C_6H_5B(OH)_2$, we were able to achieve levels of protein molar substitution markedly larger than those obtained with the $B_{10}H_{10}^{2-}$ isocyanate,⁽⁶⁾ even though the weight % was only about 0.1 because of the low boron content of $m-NH_2C_6H_5B(OH)_2$. Nevertheless, toxicity of azoproteins has been so low in our hands that even this

substitution level could possibly be adequate for therapy.⁽⁵⁾

By the use of recently reported reactions of $B_{10}H_{10}^{2-}$,⁽⁷⁾ we hope to be able to increase even these levels ten- to twenty-fold by diazotization of $B_{10}H_{10}NH_2^-$ moieties to serum proteins. For the immediate future, we will attempt such diazotizations along three pathways: 1) conversion of $B_{10}H_{10}^{2-}$ to $B_{10}H_{10}NH_2^-$ followed by diazotization and protein coupling; 2) diazotization coupling of dinitrofluoro aniline to $B_{10}H_{10}^{2-}$ followed by direct fluoro coupling; 3) monodiazotization of *p*-phenylene diamine to $B_{10}H_{10}^{2-}$ in strong acid followed by diazotization to protein in base. The resultant products will be tested for localization and toxicity studies.

Because boron has no useful radioisotopes, determination of B^{10} localization *in situ* requires sequential chemical analyses of biopsy specimens, a very cumbersome procedure for guiding therapy. To obviate this, we hope to label synthesized borono proteins with such radionuclides as I^{131} , As^{74} , Se^{75} or Hg^{203} —any one of which would permit radioscan localization of the protein even in the center of a human body.⁽⁸⁾

Uranium Chemistry

Although U^{235} possesses marked physical advantages over B^{10} (*vide infra*), uranium organic chemistry is still in its infancy so that little is available to guide us in the synthesis of appropriate urano proteins. The first requirement is for a urano organic compound, stable under physiological conditions, of low toxicity, and capable of coupling to protein. Uranyl phthalocyanine is stable, but too phototoxic for use in humans. Cyclopentadienyl uranium compounds⁽⁹⁾ so far reported are unstable to water. In our experience, uranyl chelates and porphyrins⁽³⁾ were much too toxic to be considered. Accordingly, we have attempted to make uranium macrocycles which would be sufficiently nonplanar to avoid the phototoxicity of uranyl phthalocyanine.

During the year, we have been able to synthesize the hitherto unreported uranyl derivative of hemiporphyrazine⁽¹⁰⁾ and uranyl and U(IV) tetrabenzotetraazacyclohexadecine.⁽¹¹⁾ All three were insufficiently stable for use.

In future, we will attempt to cage uranium into molecular clathrates⁽³⁾ by the covalent closure of a hexadentate ligand surrounding a uranyl ion, e.g., hexapyridyl, as suggested by recent work on the doubly tridentate chelation of uranyl ion by terpyridyl.⁽¹²⁾ Failing this, we may be able to obtain other species made stable by steric hinderance via reaction of UF_6

with alkali metal cyclopentadienes, indenenes, fluorenes or fluorenones.⁽⁹⁾

Physical Comparison of Nuclides

From a physical standpoint, it became evident during this year that U^{235} is to be favored over other nuclides on at least four counts.

1) Its specific* fission-energy yield alone is much higher than that of other nuclides,⁽¹³⁾ e.g., twelve-fold that of B^{10} . Thus, the same neutron fluence would yield much higher tumor doses and a much greater physical therapeutic ratio,† even if its nonfission energy output (neutrons, betas, gammas, etc.) is ignored.⁽³⁾

2) Its natural decay yields 95–200 keV gamma rays. This provides a convenient method for locating and determining its concentration in tumors and body tissues by conventional external scanning; the principle gamma energy of 185 keV is nearly ideal for the purpose.⁽⁸⁾

Thus, if localized in tumors by an appropriate carrier, U^{235} would provide, *de facto*, a method for determining the location of the tumor and its metastases—permitting prior decision on precisely where, when and how to irradiate with neutrons.

3) The energy and nature of U^{235} fission greatly simplifies *in situ* dosimetry. The fission-fragment ranges are so great⁽¹⁴⁾ that they readily penetrate Mylar coatings. Thus, small solid-state counters could be used *in vivo* to monitor dose during therapy. Fission-fragment energies are so large that discrimination against other radiation is easily accomplished.

Alternatively, the gamma rays emitted during fission and by the fission products are sufficiently characteristic, and their yields sufficiently well known,⁽¹⁵⁾ that dose could be monitored by external scintillation crystals with facility.

4) The relatively long range of fission fragments, ca. 50 μ in tissue, greatly simplifies the microlocalization requirements for cytotoxic effect. Thus, if localization in a tumor is chiefly an extracellular phenomenon, as seems likely^(16, 17) a B^{10} fragment travelling 3 to 6 μ would never penetrate to the nucleus of an average cell.‡ Only a few keV, of the 2.3 MeV of energy released per B^{10} capture, would even enter the cytoplasm. If the B^{10} were concentrated on the cell wall,⁽¹⁸⁾ the nucleus would still not be reached, although some 1.2 MeV would enter the cytoplasm. But a cytoplasmic cytotoxic dose is much higher than a nucleoplasmic dose. Thus, a more or less uniform distribution within the cyto-

plasm⁽¹⁹⁾ or—much better—within the nucleoplasm (e.g., via DNA incorporation⁽⁴⁾) would be needed for reasonably efficient utilization of dose.

With U^{235} , on the other hand, even extracellular localization would result in the deposition, per fission, of over 3 MeV of energy within nucleoplasm and over 130 MeV within cytoplasm. With nucleoplasmic, or even cytoplasmic localization, doses to nuclei would exceed 30 MeV. In addition, fission fragment nuclear energy transfer⁽¹⁴⁾ and emitted neutrons, electrons, and photons would increase the advantage still more, albeit only by 30% or so.

Regardless of RBE, there is little question that a fission fragment intersecting a nucleus will lead to cell death. Not only is some 16 MeV of energy deposited within the nucleus (equivalent to some 5×10^5 ionizations), but the path itself is actually damaging in a mechanical sense. Our microscopic observations of fission tracks through cells have shown *visible* tracks of scrambled cytoplasm and nucleoplasm! Thus, on purely geometric grounds, we may conservatively compute that each 10 U^{235} fissions will kill one cell nucleus—even if localized only in the extracellular fluid and ignoring any death due to cytoplasmic damage. Assuming 10^7 cells cm^{-3} , cytocide would require 10^8 fissions cm^{-3} equivalent to 270 rads of fission fragment total dose. Allowing for the usual probabilistic (i.e., exponential for high-LET radiation) nature of such processes,⁽²⁰⁾ we may equate this dose to the D_0 for mammalian cells and, in this way, arrive at a figure for 99.99% cytocide (considered adequate for tumor therapy⁽²¹⁾) of some 2700 rads.

The fair agreement of this figure with the doses usually used in x-ray or gamma radiotherapy⁽²¹⁾ is intriguing, but very possibly fortuitous. In any case, it seems conservative enough to be used as a goal. Thus, we have chosen 3000 rads to the tumor and not more than 600 rads to the most intensely irradiated nonneoplastic volume as fairly realistic goals for neutron capture therapy—a PTR of 5.

As to its disadvantages, about 125 μCi of U^{235} (60 g) must be administered for effective therapy,⁽¹⁾ equivalent to a lifetime alpha dose of 7500 rads. Although man might survive this,⁽²²⁾ it is undesirably high. Thus, compounds are needed which are not only well-localized, nontoxic and nonphotosensitizing at the $3.5 \times 10^{-3} M$ level but fairly readily excreted as well.

A question may also be raised as to the production of radioactive fission products by U^{235} . Three thousand rads to a 1-kg tumor produces only about 2.5×10^{12} fission product nuclei. This represents an activity of order of a few millicuries for the fraction which does not decay promptly within the tumor. Because of the relatively low energy yields and short effective half-

* i.e., per unit fluence and per unit molar concentration.

† PTR, the ratio of rads delivered to the tumor to rads delivered to the *most sensitive other tissue* in the body.

‡ Assuming a nuclear diameter of 10 μ and a cell diameter of 30 μ with 10- μ extracellular spaces.

lives of most fission products, they would afford a body dose negligible in comparison to the dose unavoidably given during therapy. For example, some 10^{11} Sr^{90} nuclei would be produced—equivalent to about $1 \mu\text{Ci}$.⁽¹⁵⁾ Even this could be reduced by pre-administration of chelating agents⁽³⁾ to decrease effective half-life.

The first advantage of U^{235} , the increased energy yield, can hardly be questioned since the physics of the process is so well known.⁽³⁾ In any case, the clinical cures of murine gliomas obtained with U^{235} and epithermal neutrons⁽¹³⁾ show that it is therapeutically effective.

During the past year, we have been able to check experimentally the second and third of its potential advantages.

Using the neutron system described elsewhere,⁽¹⁾ a 35-cm^3 "tumor" at $3.5 \times 10^{-3} M$ U^{235} , in a head $3.5 \times 10^{-4} M$ in U^{235} was scanned externally using the U^{235} 110- and 185-keV gamma lines. Even with relatively crude equipment, this gave scans which compared favorably with those obtained using Tc^{99m} , Hg^{197} , I^{131} , etc.⁽⁸⁾ The concentration used, if present uniformly throughout a 70 kg human, would represent a total activity of $125 \mu\text{Ci}$ —quite within the 25–10,000 μCi range now used for scanning.⁽⁸⁾

A solid-state Si detector, potted in epoxy resin and provided with a $5\text{-}\mu$ Mylar window, yielded a usable spectrum of fission fragments with good alpha discrimination when exposed within the "tumor" during irradiation at 50 μamp beam current. A scanning system located outside the head also gave a useful scan of U^{235} fission products within the "tumor" immediately after irradiation. The 307-keV line of 14.6-min Tc^{101} proved most convenient for short irradiations, and the 294-keV line of 33-hr Ce^{143} for longer ones. Both systems functioned better with increasing beam currents, so they should function very well at 2000–4000 μamp .⁽¹⁾

The fourth aspect, that of microdosimetry, presently rests on the success of U^{235} with murine gliomas⁽¹³⁾ versus the failure of the well-localized B^{10} Evans blue⁽³⁾ for the same tumor. Since the reasons for this difference may lie in another direction,^(3, 5) we will, in the future, attempt a more direct comparison of U^{235} and B^{10} by combining irradiation with U^{235} fission fragment and B^{10} alpha radioautography.

CONCLUSIONS

Therapeutically useful compounds for the neutron capture therapy of somatic tumors are not yet at hand. Results during the past year, however, indicate that compounds are within reach. For U^{235} , marked physical advantages over B^{10} have been observed. How-

ever, chemical problems are still great and unlikely to be solved without considerable development of the field of organouranium chemistry.

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THE LOCALIZATION OF DYES AND AZOPROTEINS IN TUMORS

Norman A. Frigerio and Michael J. Shaw

PURPOSE AND METHODS

For effective neutron-capture therapy, it was necessary to know whether a massive dose of labeled protein would localize in tumors, i.e., doses large enough to yield a tumor concentrations of at least $3.5 \times 10^{-3} M$ in the nuclide undergoing neutron capture.⁽¹⁾ A priori there was reason to believe that a massive dose would not localize like a tracer dose since it might more drastically perturb the physiology of the system.

It was also of importance, from the standpoint of therapy, to use spontaneous or induced, but not implanted, tumors. Mechanical implantation could easily give false localization which would not be equivalent to localization in spontaneous tumors.

It was first important to determine quantitatively the organ, tissue, and tumor concentrations of the fissionable nuclide as a function of time after injection. Equally important, was the need to determine the ratio of its concentration in tumor to that in the blood, since blood concentration is the critical limiting therapeutic factor.⁽¹⁾

The localization of serum proteins in inflammations was noted as long ago as 1882.⁽²⁾ This has since been amply confirmed by qualitative observations, but few, if any, other quantitative measurements have been made.⁽³⁾

Duran-Reynals, in early semiquantitative work, showed that massive amounts (of the order of 7 milligrams injection dose) of horse, pig, and chick sera localized strongly in murine lung and mammary tumors.⁽⁴⁾ The localization of labeled proteins, such as I^{131} and I^{125} human serum albumin and fibrinogen, in tracer amounts has since been extensively studied,⁽⁵⁾ but these studies have not yielded information in terms of the absolute concentrations obtainable with large doses.

Mego and McQueen have demonstrated that arson-azoproteins will localize in implanted murine brain tumors.⁽⁶⁾ However, being principally concerned with the development of a tumor-scanning technique, they used only trace amounts of the labeled protein. Furthermore, they used cerebral transplanted tumors which one cannot assume will exhibit the same localization properties as spontaneous tumors.

Localization of proteins throughout murine tumors was observed by Duran-Reynals.⁽⁴⁾ This was further substantiated by Ghose,⁽⁷⁾ who fluorimetrically observed the selective localization of labeled proteins within the cytoplasm of the tumor cell.

Sulfonic acid vital dyes, such as Evans blue and

trypan blue, are known to localize in inflammations.⁽⁸⁾ Several authors have also noted their localization in tumors, but they have likewise demonstrated that localization of sulfonic acid dyes occurs neither uniformly nor intracellularly, but rather at the growing edge of the cell and extracellularly.⁽⁴⁾ Kruger observed a brain tumor localization of boronic acid analogues of these dyes which occurred exclusively at the growing edge of the tumor.⁽⁸⁾ For possible application to neutron capture therapy, it is evident that intracellular localization is most likely to result in cytocide.⁽¹⁾ Therefore, we concentrated on proteins, rather than dyes, since the results cited above led us to expect more uniform, and possibly intracellular, protein localization.

One class of dyes, the phthalocyanine tetrasulfonates seems not only to localize in tumors but to permeate them as well.⁽⁹⁾ At any rate, the U^{235} analog was successful in the treatment of implanted murine brain tumors.⁽⁹⁾ Their rigid planar structure results in a molecule whose effective size is more nearly that of a protein than the usual dyes, and this structural peculiarity may be responsible for their success. It was of interest, therefore, to examine the question of their localization in spontaneous somatic tumors rather than simply the implanted brain tumors used before.

At present, two nuclides, B^{10} and U^{235} , seem to have possible application to neutron capture therapy. Either of them might be coupled to a protein carrier. We have successfully diazotized *m*-aminophenyl boronic acid to serum albumin. Because of technical difficulties in analyzing boron in tissue, however, we chose to use its analog, *p*-amino-phenyl arsonic acid in our localization studies.

PROGRESS REPORT

Mouse serum albumin (MSA) was recovered from whole blood in 0.1 *M* sodium citrate by half saturation with ammonium sulfate, centrifugation, dialysis, and lyophilization.⁽¹⁰⁾ Para-arsanilic acid was then diazotized to MSA by the method of Mego and McQueen.⁽¹¹⁾ Neutron activation analysis showed a bound As content of 172 moles of As/mole of azoprotein. A toxicity study indicated that a safe amount of azoprotein to inject into even an unhealthy mouse was 15 mg in 0.1 ml of solution.

Fifteen milligrams of azoprotein were injected into both healthy mice and mice with spontaneous tumors. The latter were obtained by a periodic culling of the animal colony. They were sacrificed at various time

intervals and their organs, including tumors, were analyzed for arsenic by means of neutron activation using epithermal neutrons to minimize sodium activation via neutron capture. Tissues were counted using a calibrated pulse-height multichannel analyzer.⁽¹¹⁾ Calculations were done by a GE 225 computer with a FIZ MOP program. Results are presented in Table 70, showing the concentrations of injected arsenic in various organs of mice sacrificed 24 hours after injection.

Seventy-two hours after injection, a single, whole bone tumor showed a concentration of 1.25×10^{-4} M, while blood showed no detectable arsenic, i.e., a tumor-to-blood ratio of almost infinity. Since a tumor-to-blood ratio of about 10 would yield effective neutron capture therapy,⁽¹⁾ the results can be said to be very encouraging.

Rate curves of our data indicated an exponential decrease of arsenic concentration in the blood which asymptotes to zero at about 24 hours. But a quasi-static tumor concentration is maintained at least as long as 72 hours from injection. Tumor concentrations obtained were well within an order of magnitude of that needed for effective neutron capture therapy.⁽¹⁾

Our preliminary data indicate tumor/skin ratios of 1 to 1 at 24 hours after injection. This, too, might be acceptable from the therapeutic standpoint, since intermediate-range neutrons yield their highest doses at penetrations of about 4 cm from the skin. Thus lower tumor/skin ratios than tumor/blood ratios might be acceptable.⁽¹⁾

We have also investigated foreign azoproteins, e.g. arsonazo bovine serum albumin. Arsenic concentrations in the skin and blood of healthy mice decreased more rapidly than with arsonazo MSA. Thus, it is possible that foreign azoproteins may prove more useful than native ones. We will, in the future, determine the tumor localizations of such foreign arsonazo proteins.

A small series of mice bearing spontaneous tumors of varied types was also injected intravenously with Co⁶⁰-labeled cobalt phthalocyanine tetrasulfonate, prepared in the same manner as the uranyl analog.⁽⁹⁾ Amounts injected were adjusted to yield a final concentration of 10^{-3} moles per kilogram mouse. Animals were sacrificed serially after injection; organs, tissues and tumors were removed and counted in a calibrated NaI 400-channel gamma-counting system.⁽¹¹⁾ Results for typical 48-hour sacrifices are shown in Table 71.

Blood and brain cleared so rapidly that no compound could be found in them at 48 hours, a very desirable situation from the standpoint of neutron-capture therapy. Localization was high in kidney, liver, and tumor, and moderate in other tissues. Thus, the tumor therapeutic ratios for the three most critical

TABLE 70. THE LOCALIZATION OF ARSONAZO MOUSE SERUM ALBUMIN IN MICE SACRIFICED 24 HOURS AFTER INJECTION

Tissue	Concentration in healthy mice, moles kg ⁻¹ × 10 ⁻⁴	Concentration in tumor mice, moles kg ⁻¹ × 10 ⁻⁴
Blood	0.19	0.48
Skin	1.30	2.60
Liver	8.40	6.30
Kidney	10.70	10.70
Brain	0.16	0.10
Muscle	0.70	0.60
Spleen	10.80	4.70
Intestines	1.89	1.35
Heart	1.53	1.18
Whole tumor	—	2.01
Growing edge of tumor	—	9.96
Anaerobic core of tumor	—	2.65

TABLE 71. LOCALIZATION OF Co⁶⁰-PHthalocyanine TETRAsulfonate IN SPONTANEOUS MURINE TUMORS

Tissue	Concentration, moles kg ⁻¹
Blood	<10 ⁻⁶
Brain	<10 ⁻⁶
Heart	9 × 10 ⁻⁶
Spleen	1.1 × 10 ⁻⁴
Intestine	1.6 × 10 ⁻⁴
Skin	1.6 × 10 ⁻⁴
Tumor	9 × 10 ⁻⁴
Liver	1.0 × 10 ⁻³
Kidney	1.6 × 10 ⁻³

Values for mice sacrificed 48 hours after injection of sufficient compound to yield an initial mean concentration of 10^{-3} moles per kg total body weight. Values are means for 3 to 6 mice, and extremes lay within $\pm 20\%$ of the means given.

tissues, blood, skin, and muscle, were almost infinity, 4.9, and 3.1, respectively—all quite useful from a therapeutic standpoint.⁽¹⁾

CONCLUSIONS

Both arsonazo mouse serum albumin and cobalt phthalocyanine tetrasulfonate anion have been found to localize well in spontaneous somatic murine tumors. Concentrations and tumor/tissue localization ratios obtained would be adequate for neutron-capture therapy should U²³⁵ analogs follow the same pattern, which seems very probable on chemical grounds. Maximum ratios are obtained over relatively long periods, i.e., 24–72 hours, so that dose-rate problems⁽¹⁾ could be avoided. Results obtained indicated that even more favorable ratios and concentrations are to be expected from further development along these lines.

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BENZENEBORONIC ACIDS. I. INTERACTIONS WITH TISSUE AND USE FOR CATALASE VISUALIZATION ON SOLID MATRICES

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PURPOSE AND METHODS

In testing boronic acids for possible use in neutron-capture therapy⁽¹⁾ we noted that borono Evans blue was oxidatively hydrolyzed *in vivo* to free H_3BO_3 .⁽²⁾ Further work showed this hydrolysis to be peculiar to the tumor-bearing mice used, and could not be confirmed for other laboratory mammals or for humans.⁽³⁾

The first possibility was that hydrolysis was catalyzed enzymatically, and a search for such an enzyme began.⁽²⁾ At the same time, it was known that benzenboronic acids are oxidized to borate esters by H_2O_2 and the esters subsequently hydrolyzed to phenol and H_3BO_3 ,⁽⁴⁾ thus:

As a consequence of the presence of catalase, the steady state concentration of H_2O_2 in normal tissues is probably too low to promote reaction (1). Tumor tissues, however, are known to be generally low in catalatic activity,⁽⁵⁾ and hence, probably high in H_2O_2 . We undertook, therefore, an investigation of the interrelationships of H_2O_2 , catalase and boronic acids to determine whether the effects observed⁽²⁾ could be attributed to tumor H_2O_2 .

On theoretical grounds, a colorless nitrobenzenboronic acid should undergo reactions (1) and (2) and, in strong base, ionize to a bright yellow nitrophenoxide anion, thus:

This proved to be the case and was made the basis of a successful method for detection of catalatic activity on solid matrices.

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PROGRESS REPORT

Hydrolysis of Boronic Acids

Samples of mouse liver, heart, muscle, kidney and skin were homogenized 1:1 in pH 7.4 0.05 *M* phosphate buffer. Similar homogenates of spinach and mother-in-law tongue leaves were also made. The resultant homogenates were made 10^{-3} *M* in benzenboronic acid and incubated at room temperature for periods up to 24 hours. Serial aliquots were taken, and each aliquot was analyzed for phenol by the Folin-Ciocalteau method,⁽⁶⁾ along with control aliquots free of boronic acid, and aliquots containing one mole of H_2O_2 per mole of boronic acid. The latter clearly gave one mole of phenol per mole of boronic acid, but the homogenates showed no evidence (i.e., <0.1%) of hydrolysis. We concluded that neither plant nor animal tissues normally contains either enzymatic activities or H_2O_2 sufficient to bring about significant oxidative hydrolysis of benzenboronic acids.

With three spontaneous tumors, however, one from a rat and two from mice, an oxidative hydrolysis equivalent to a H_2O_2 concentration of about 10^{-4} *M* was observed. So far only these three tumors have been tested, each as a homogenate in phosphate buffer containing 0.05 *M* NaN_3 to inhibit any catalase. However, all three showed equivalent H_2O_2 concentrations at least 100-fold those in other tissues from the same, or any, animal (which, in fact, showed no detectable activity). It seems very possible, then, that H_2O_2 or some similar peroxide is present in high concentrations in tumors and that its activity on boronic acids accounted for the failure of neutron-capture therapy efforts with borono Evans blue.⁽²⁾

Method for Visualization of Catalatic Activity on Solid Matrices

In the light of equation (3) *m*-nitrobenzenboronic acid was synthesized⁽⁷⁾ and purified by recrystalliza-

tion from water until the extinction coefficient of a solution in saturated aqueous NaHCO_3 at $400\text{ m}\mu$ was constant at $30\text{ liter mole}^{-1}\text{ cm}^{-1}$, and the melting point was constant at 277°C (Lit., $275\text{--}276.5^\circ\text{C}$).

Paper strips containing $20\text{ }\mu\text{l}$ of catalase solution were air dried, sprayed with 0.05 M H_2O_2 , air dried again and sprayed with 0.05 M *m*-nitrobenzeneboronic acid in 2% aqueous NaOH (or in saturated aqueous NaHCO_3). Both solutions were made fresh each day as a matter of caution, but proved usable even after several days' storage at room temperature.

Regions containing catalase were white against a bright yellow background and the colors remained stable for at least a month; catalase regions yellowed slowly after that. Catalase was clearly detectable down to less than 0.001 perborate unit⁽⁸⁾ on a 1-cm^2 spot. The same value of sensitivity was also obtained with electrophoretograms of normal and acatalatic mouse blood.* Sensitivity was so high that catalatic activity was observed in the hemoglobin region. This could be due either to the weak catalatic activity of hemoglobin itself,^(9,10) to association of some of the blood catalase with hemoglobin, or to multiple catalases of differing mobility.

CONCLUSIONS

1. No activity capable of oxidatively hydrolyzing benzeneboronic acids was found in either plant or animal tissue homogenates.

2. However, such activity was found in all three spontaneous tumor homogenates tested. This could ac-

* Courtesy of Dr. Robert N. Feinstein.

count for previous failures of neutron-capture therapy which involved the use of boronic acids.

3. The reaction of *m*-nitrobenzeneboronic acid with H_2O_2 has been used to develop a method of visualizing catalatic activity on paper and similar solid matrices. Sensitivity on paper was at least 0.001 perborate units cm^{-2} .

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BENZENEBORONIC ACIDS. II. USE OF META-NITROBENZENEBORONIC ACID FOR THE DETERMINATION OF H_2O_2 AND H_2O_2 -ENZYME SYSTEMS

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PURPOSE AND METHODS

The importance of boronic acids in schemes for neutron-capture therapy,^(1,2) coupled with their probable oxidative hydrolysis by tumor tissues^(3,4) prompted us to undertake an extensive investigation of the interrelationships of boronic acids with the components of tissue which might be responsible for such hydrolysis. The reactions of importance are probably⁽⁴⁾

Because reaction (2) proceeds fairly rapidly under ordinary physiological conditions, and because tissues contain many esterases capable of catalyzing it even more rapidly, attention was focused on (1). As a preliminary to such studies, it appeared necessary to develop methods for determination of H_2O_2 and for the chemical and enzymatic systems involved in its production and destruction. Further, because our primary interest is in the boronic acids, it seemed advisable to develop methods based on their chemical reactions. We have predicated our approach on the reactions of nitrobenzeneboronic acids which yield colored nitrophenoxide ions⁽³⁾ by ionization following reactions (1) and (2):

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FIG. 95.— E_{400} as function of H_2O_2 added, Bausch and Lomb Spectronic 20 spectrophotometer.

The extinction coefficients of meta and paranitrophenoxide ions at 400 $m\mu$ are 1500 and 17,500 liter mole⁻¹ cm⁻¹, respectively.^(5, 6) While the latter would provide the most sensitive method for H_2O_2 yet to be described,⁽⁷⁾ synthesis of the required para nitrobenzeneboronic acid has so far proved unfeasible. We have, in the interim, utilized the meta analog⁽³⁾ which has provided methods of adequate sensitivity.

A mechanism has been proposed for reaction (1) which involves coupling of H_2O_2 to the electron-deficient locus of boron.⁽⁸⁾ This mechanism became untenable upon our finding that meta nitrobenzene *arsonic* acid reacted nearly as rapidly as the boronic analog despite the lack of a similar locus on the arsenic.⁽⁹⁾ Nevertheless, reactions (1) and (2) proceed well in practice so that the lack of a mechanism has not impeded our empirical development of appropriate methods.

PROGRESS REPORT

Determination of H_2O_2 in Aqueous Solution

To 3.0 ml of H_2O_2 containing solution in a 13 × 100-mm test tube, we added 1.0 ml of 5×10^{-3} M meta nitrobenzeneboronic acid⁽³⁾ in saturated aqueous $NaHCO_3$, and 5 minutes was allowed for completion

of reaction (1). Then, 1.0 ml of M NaOH was added, 5 to 20 minutes allowed for completion of reactions (2) and (3), and the resulting yellow color was read at 400 $m\mu$ in a Bausch and Lomb Spectronic 20 spectrophotometer. Results are presented in Figure 95. The ex-

TABLE 72. MOLAR EQUIVALENTS OF VARIOUS REAGENTS IN THE META NITROBENZENE BORONATE DETERMINATION OF H_2O_2

Reagent	Quantity, μ moles*	E_{400}	Mole equivalence
H_2O_2	3.0	0.740	1.00
Ascorbic acid	3.0	0.200	0.27
$NaBO_3$	3.0	0.740	1.00
Cysteine	3.0	0.010	0.014
Succinic peroxide	3.0	0.585	0.79†
Benzoyl peroxide	3.0‡	0.0	0.0

* To the indicated number of μ moles dissolved in 3.0 ml water, 1.0 ml of 5×10^{-3} M meta nitrobenzeneboronic acid in saturated $NaHCO_3$ was added. Between initial solution and this addition sufficient time was allowed (less than 30 minutes in every case) to permit complete autoxidation, if any. Five minutes after the addition, 1.0 ml of NaOH was added and E_{400} read periodically until no more change was observed (less than 20 minutes in every case).

† A commercial product whose purity may be suspected. Theory would lead one to expect an equivalence of 1.0.

‡ In suspension.

FIG. 96.—Catalase assay

tion coefficient observed corresponded to that expected from the literature value⁽⁵⁾ for meta nitrophenoxide ion and the stoichiometry of reactions (1), (2), and (3). Maximum sensitivity was 1.5×10^{-7} moles H_2O_2 , corresponding to an initial concentration of $5 \times 10^{-5} M$. Reducing each of the volumes to one-fifth of the values given above and reading in a 1.0-ml cuvette on a Beckman DU spectrophotometer gave a sensitivity of 3×10^{-8} moles H_2O_2 . Blanks, in both cases, were negligible.

The reaction was also promoted by substances which release H_2O_2 in aqueous solution (e.g., perborate), by certain organic peroxides, and by substances which autoxidize aerobically to produce H_2O_2 . The H_2O_2 molar equivalences of substances, found by substitution for H_2O_2 , are listed in Table 72.

The method was found to work equally well at pH's between 2-14. Up to 120 minutes were needed for completion of reaction (1) at pH values of 1 or 13. Within its optimum pH range, 6 to 9, 10 minutes was adequate time for completion of (1).

Variations of $\pm 20\%$ in reagent concentrations had no effect on the determination, nor did tissues, biological fluids or any of the usual biochemical buffers interfere. Finally, the method proved extremely reliable, reproducible, and stable in practice. Thus, it is directly applicable to biochemical determinations with distinct advantages over the iodine, titanium, or amine methods which have been used heretofore.⁽⁷⁾

Application to the Assay of Catalase

Since the method above measures H_2O_2 , it should be useful for enzymatic assays where H_2O_2 is produced (e.g., oxidases) or consumed (e.g., catalase, peroxidases). Catalase not only destroys H_2O_2 but is destroyed by H_2O_2 at low concentrations. Thus, response is logarithmic rather than linear, even when a fixed reaction time is used. A series of tests and measurements led to the method given below. In principle, a known quantity of H_2O_2 is reacted with unknown catalase for 10 minutes and the reaction is stopped by addition of the catalase inhibitor N_3^- . The H_2O_2 remaining is determined by the procedure above.

A solution containing 3.2 ml of catalase was added to 1.0 ml of $3 \times 10^{-3} M$ aqueous H_2O_2 , usually in 0.05 M phosphate buffer at pH 6.85. Ten minutes later, 0.5 ml of $5 \times 10^{-3} M$ aqueous NaN_3 was added, followed immediately, by 1.0 ml of $5 \times 10^{-3} M$ meta nitrobenzeneboronic acid in saturated aqueous NaHCO_3 .

After 5 minutes, 0.3 ml of 0.5 M NaOH was added, and E_{400} was read from 5 to 10 minutes afterward. The results are presented in Figure 96. The catalase solution used was analyzed for activity in perborate units,⁽¹⁰⁾ and confirmed through the courtesy of Dr. Feinstein.

Once again, sensitivity was increased by a factor of five by conducting the operation in 1.0-ml Beckman DU cuvettes.

CONCLUSIONS

1. A colorimetric micromethod has been developed for the determination of H_2O_2 based on the oxidative hydrolysis of meta nitrobenzeneboronic acid and the spectrophotometric determination of the resultant meta nitrophenoxide ion at 400 $m\mu$. The method is sensitive to 3×10^{-8} moles (1 μg) of H_2O_2 , stable, rapid, reproducible, convenient, and applicable to biochemical analytical requirements.

2. The method has been extended to the determination of catalase with a sensitivity of about 10^{-3} perborate units. It will be extended to the determination of oxidases and peroxidases and in future studies.

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MICRODETERMINATION OF URANIUM IN TISSUE

Norman A. Frigerio and Donald Doran*

FIG. 97.—Determination of uranium in aqueous solution

diagnostic laboratories. Accordingly, we developed a simple colorimetric micromethod that uses only equipment and reagents available in any clinical laboratory. Details of the development of the method have been described elsewhere.⁽³⁾

PROGRESS REPORT (FINAL)

Because of its sensitivity, ubiquity and the low cost of the reagent, the reaction of the uranyl ion with the ferrocyanide ion was finally chosen. This reaction produces a deep red uranyl ferrocyanide, which is easily read colorimetrically. The compound is inherently insoluble, but the transition from solution to solid phase is slow. After 2 hours, only 49% of the compound was removed by centrifugation at 144,000 *g*.

FIG. 98.—Extraction procedure

PURPOSE AND METHODS

Accompanying the utilization of U^{235} for neutron-capture therapy of tumors,⁽¹⁾ a simple method is needed for the determination of uranium in tissue. While a number of methods exist,^(2,3) none of them is sufficiently simple in practice or in its equipment requirements to permit its use in the average hospital laboratory. We have been able to use gamma-scintillation counting of the characteristic U^{235} lines at 110 and 185 keV to determine concentrations as low as 10^{-8} moles in 1-gram samples of tissue. However, the sophisticated equipment required is not standard in most

After addition of the reagents, the solution is stable up to 24 hours. It has a maximum light absorption at 400 $m\mu$ on the Bausch and Lomb Spectronic-20 colorimeter. The absorption followed Beer's Law over the surprisingly large range of 0 to 2.0 units of optical density. The response for an aqueous solution is shown in Figure 97. Readings were taken at 400 $m\mu$ 15 minutes after addition of reagents. Final volume was 5 ml in 1-cm cuvettes.

These results led us to the development of the analytical method for uranium in tissue outlined below (Figure 98):

1) Tissue samples are ashed in covered crucibles at 700°C for 2 hours, or with a Fisher burner by hand.

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2) After cooling, add 2 ml concentrated HNO_3 , cover with a watch glass and reflux for about 10 minutes on a hot plate.

3) After cooling, wash quantitatively into a 60-ml separatory funnel containing 15 ml ethyl acetate and 15 ml of a nearly saturated aqueous solution of $\text{Al}(\text{NO}_3)_3$. Shake, remove the ethyl acetate layer, re-extract with 15 ml of fresh ethyl acetate. Combine extracts in a 50-ml beaker and evaporate to dryness on a hot plate, then continue to heat for another 20 minutes.

4) Extract with water, filtering off any insoluble residue while transferring the extract to a cuvette. Make volume up to 4.0 ml, add 1.0 ml of 0.05 M $\text{K}_4\text{Fe}(\text{CN})_6$.

5) Mix, wait 10 minutes, read at 400 m μ .

The results of this procedure, applied to known added amounts of U per gram of tissue, are shown in Figure 99. In this example, beef liver was used as the tissue because, from experience, it contains the highest concentrations of potentially interfering elements (e.g., Fe, Cu, etc.) of any tissue.

By using one-fifth the above volumes and a 1.0-ml, 1-cm Beckman DU cuvette, the sensitivity was increased exactly by a factor of five; thus, as little as 4 micrograms U could be determined conveniently.

CONCLUSION

A simple, rapid, and inexpensive method is described for the microdetermination of uranium in tissue.

FIG. 99.—Determination of uranium in tissue

The method involves dry ashing, organic extraction, and colorimetric determination of uranyl ferrocyanide at 400 m μ .

On the basis of the recovery of uranium from beef liver (a tissue with many interfering inherent ions), this procedure should be applicable to any tissue.

It will determine 4 micrograms of uranium.

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RESPONSE OF PROLIFERATIVE CELL SYSTEMS TO ASCITES TUMOR GROWTH

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PURPOSE AND METHODS

The study was undertaken to compare the effect of starvation and the effect of rapidly growing neoplastic mast cell tumors on:

- a) Life span of mouse
- b) Cell proliferation in
 - 1) rapidly proliferating tissues (intestine)
 - 2) slowly proliferating tissues (cornea)
 - 3) haematopoietic tissues (spleen, liver)
- c) Histological change in grossly altered tissue (kidney), as shown by autopsies and tissues which may distribute tumor cells (blood)
- d) Histological characteristics of tissue where tumor invasion (mesentery) and metastasis (lung) are most likely to occur.

DBA/2 mice were divided into two groups; those

from which food was removed and those injected with 2×10^6 neoplastic mast cells. The cells are injected into the peritoneal cavity and grow as ascites. Fifteen animals from each group were sacrificed between days 1 and 23, and the remaining fifteen animals were allowed to die. Animals in the latter category contribute data toward the percent survival curve with respect to time.

Animals which were sacrificed were injected with 20 μCi of tritiated thymidine and 160 μg of colcemide 2 hours before killing with ether. At sacrifice, samples of the following tissue were taken: intestine, liver, kidney, lung, spleen, cornea, blood, and esophagus. Squash preparations were made of crypts and cornea, and all tissues were sectioned. The number of labeled cells, the number of mitoses, and the number of labeled mitoses were scored in squash preparations and the labeled

mitosis/mitosis ratio was determined. Sections show histological changes and relative position and frequency of labeled cells.

PROGRESS REPORT

The neoplastic mast cell tumor P815Y grows in the peritoneal cavity of the DBA/2 mouse. The increase in both cell number and ascitic fluid volume causes significant abdominal distention. Sometime after the injection of cells into the peritoneal cavity, depending in part on the size of the inoculum, the animal dies. Autopsies indicate notable changes in several tissues including intestine, liver, spleen, and mesentery. The causes of death are not known but alteration in the general nutrition is undoubtedly a contributing factor. It is not clear in which way starvation and the alteration in nutrition accompanying tumor growth differ in the way they affect proliferative cell systems. For example, changes in intestinal cell turnover in tumor-bearing animals might be due to altered food intake, whereas in some tissue such as the reticuloendothelial system, increased proliferation might occur despite changes in nutrition. The question of whether proliferation occurs in tissues of tumor-bearing animals is still not clear.^(1, 2)

The alteration in general nutrition is not the only disturbance. Animals whose peritoneal cavities have been frequently rinsed with saline to remove tumor cells after the 11th day show considerable life shortening. However, rinsing increased survival in these animals by about a factor of two and the survival curve is less steep.

In order to follow cell proliferation and histological changes, animals were sacrificed on a near daily basis,

and tissues were removed for examination. Two hours before sacrifice, animals were injected with 20 μ Ci of tritiated thymidine and 160 μ g of colcemide. This procedure permits an identification of the size of the proliferative pool as well as a measure of the relative rate of movement of cells from the DNA synthetic period to mitosis. Tissues selected for studies of cell proliferation and histological changes have been previously noted.

The slope of the sigmoid survival curve in both groups is similar except that the starved animals begin dying earlier. All starved animals die between days 7 and 14; all tumor-bearing animals die between days 20 and 26.

The number of white cells in tumor-bearing animals rises steadily from several days before neoplastic mast cells appear in the blood (day 11) until death. After the number of white cells begins to increase and the number of polymorphonuclear leucocytes also increases, the number of lymphocytes begins to fall steadily with both changes continuing until death. There is no notable change in the number of white cells in starved animals preceding death, or in the differential counts from these animals.

The crypts show an immediate reduction in the number of labeled cells and the number of mitotic figures. The change in the labeled mitosis/mitosis ratio, which is not immediate, indicates that cells in the S period require a longer period of time to reach mitosis.

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GENERAL OBJECTIVES OF THE PROGRAM ON THERAPY OF POISONING BY RADIOACTIVE AND NONRADIOACTIVE METALS

Arthur Lindenbaum and Marcia W. Rosenthal

The general purpose of this program since its inception has been the development of new approaches to the therapy of poisoning by radioactive and nonradioactive metals. Much attention has been given to the bone and liver because of the importance of these organs in the accumulation and toxicity of polyvalent metals. An early experimental approach introduced the use of non-toxic colloidal metal salts as carriers to prevent bone deposition by removing circulating radioelements from the blood and body. A continuing major effort has been the development and use of complexing agents, as exemplified by such chelating agents as citrate, a natural metabolite, and the synthetic polyamino acids, for therapeutic purposes. This approach depends upon the selection of chelating agents capable of removing tissue-bound toxic metals by forming soluble complexes, which are then excreted. The successful use of chelation therapy to inactivate a toxic nonradioactive metal *in situ*, without necessarily promoting increased excretion, has also been demonstrated. Another approach has been to alter normal physiological processes (e.g., blocking the Krebs cycle to allow citrate accumulation *in vivo*) to promote increased metal excretion.

In the course of this work new problems and outlooks have developed which indicate the importance of allied experimental areas to achieve the over-all objectives of this program. Problems and areas of emphasis at present may be briefly outlined as follows:

1) Divalent cations in the body, notably calcium, compete with the toxic metal for the chelating agent. For the removal of alkaline earth metals in particular, reducing this competition by the development of chelating agents with low relative affinity for tissue cations is essential.

2) Under physiological conditions there are significant differences in physical state between compounds the rare earth and transuranic elements (e.g., plutonium) and those of the alkaline earth metals (e.g., strontium or radium). Shifts from the soluble to col-

loidal state in tissues, especially for the transuranic elements, result in: a) differences in the sites of deposition within tissues and within individual organs; b) differences in extra- and intracellular deposition due to phagocytic action; c) shifts in urinary and fecal excretion ratios; and d) differences in the effectiveness of therapy. These, as well as other factors, must be considered in devising more effective means of radioelement removal.

3) As a result of a meeting on "Colloids and Particulates in Tissues," held in the Division of Biological and Medical Research, Argonne National Laboratory in January, 1965, at the invitation of this group, it became obvious that our understanding of many aspects of the metabolism of colloids, particulates, and macromolecules is seriously deficient. Much remains to be understood about the following: factors influencing movement of particles within tissues of the reticulo-endothelial system; relative phagocytic activity in different RE tissues; factors affecting particle translocation and excretion in the body; and therapeutic manipulation to remove foreign particles. With tools now at hand, several of these problems appear to be open to attack. These tools consist of transuranic element tracers, such as plutonium, carefully characterized with respect to particulate properties, combined with techniques adapted from such diverse fields as electron microscopy, autoradiography, light-scattering, density gradient centrifugation, measurement of phagocytic indices, etc. The use of these tools should not only contribute to our basic information but also to the development of more effective therapeutic procedures for the removal of hydrolyzable toxic elements.

4) Differences in the retention sites of different classes and forms of radioelements and the shortcomings of chelating agents in removing radioelements from tissues have led to other therapeutic approaches. One involves modification of the physical properties of existing chelating agents to facilitate contact with

polymerized and phagocytized radioelements. Other approaches involve modification of phagocytic activity or bone metabolism. Another long-term approach is to attempt to improve our knowledge of toxic cation uptake and removal in tissues by studying the normal

process of calcium uptake and depletion in the calcifying matrix of preosseous cartilage. Such efforts have the virtue of yielding information of basic, as well as practical, importance and are becoming increasingly important segments of the over-all therapy program.

METABOLIC AND THERAPEUTIC STUDIES OF PLUTONIUM

Marcia W. Rosenthal and Arthur Lindenbaum

PURPOSE AND METHODS

Plutonium is representative of the heavy, polyvalent, easily-hydrolyzed radioelements which polymerize and form colloidal and insoluble polymers under physiological conditions. As such, it deposits both in bone (where its alpha radiation makes plutonium one of the most effective carcinogenic agents known) and in the liver. In both tissues the particulate properties of the plutonium affect the pattern of uptake and the extent of retention, with or without therapy. Two specific problems are receiving attention at present: a) the removal from bone of the fraction of deposited metal (about one-half) which cannot be removed by the most effective polyamino acid chelating agent, diethylenetriaminepentaacetic acid (DTPA); and b) the removal from liver of the polymerized plutonium which is not removed by DTPA and which is presumably intracellular. In addition to the work reported below, a parallel investigation of the uptake of cations by preosseous cartilage is part of a broad and basic attack upon the first problem. The methods in use are generally as outlined earlier.⁽¹⁾

PROGRESS REPORT

Measurement of Colloidal Properties of Plutonium in Solutions and in vivo

In order to relate the colloidal state of plutonium to its tissue distribution, toxicity, and therapy, it is necessary to have a better method for characterizing its particulate properties than the ultrafiltration technique presently available.⁽²⁾ The measurement of particle sizes of plutonium in injection solutions is at present being attempted by means of light-scattering and density gradient centrifugation. Visualization and measurement of plutonium-containing particulates in tissues is also being attempted by means of electron microscopy and autoradiography. An initial study, characterizing plutonium alpha tracks at high resolution, has indicated the possibility of precise intra- and extracellular localization of plutonium particles below the resolving power of the electron microscope.⁽³⁾ Elec-

tron microscopic work attempting to visualize the deposition and intrahepatic movement of particulate plutonium in liver is now in progress to extend earlier work⁽⁴⁾ with other techniques.

The measurement of the deposition of radiocolloids in bone marrow, relative to that in other reticuloendothelial organs, and in hard bone, and the variation of these deposits with the physical state of the plutonium is important both in therapeutic and dosimetric studies. The preceding report⁽¹⁾ described the use of a method of physical separation of marrow (containing plutonium) from hard bone in a standardized 9.0-mm mid-shaft segment of mouse tibia. Although this technique does not measure the total amount of plutonium in the marrow of the whole animal, it is a reproducible index of marrow plutonium for comparative purposes. The initial distribution of three different forms of plutonium of increasing degrees of polymerization (90, 30, and 17.5% ultrafilterable, respectively) 6 days after injection into mice was reported⁽¹⁾ and served as another indication of the physical state of the administered plutonium. With increasing polymerization of the plutonium, the fraction deposited in bone decreased and the fraction in liver and spleen increased; bone marrow content also increased, but was intermediate in mice receiving the most highly polymerized form, presumably because of the efficiency of the liver in removing this form from the blood.

Retention data at 90 days are now available. The liver content of plutonium was reduced in all groups, but the degree of reduction (91%, 46%, and 34%, respectively) decreased with increasing polymerization of administered plutonium. In all three groups the content of plutonium in the spleen was unchanged. The marrow concentration of plutonium remained the same in mice receiving the two most polymerized forms of plutonium, but in those receiving the monomeric form there was a greater than twofold increase at 90 days. This increase may be the result of osteoclastic activity with transference of plutonium bound to the surfaces of trabecular bone into macrophages of the marrow, as described earlier.⁽⁵⁾ In support of this, there was a

significant drop in the plutonium content of the *whole* femur between 6 and 90 days, but no loss from the bone of the tibial shaft segment, which contains no trabeculae. Mice receiving the two most polymerized forms of plutonium showed little or no loss of isotope from the femur or tibial shaft. These experiments demonstrate again the long-term retention of polymerized plutonium in the liver, and in addition demonstrate the permanence of the retention of all forms of administered plutonium in the bone marrow.

DTPA Therapy in Plutonium Poisoning

Long-Term Effects.—Malignant bone tumors, primarily osteogenic sarcomas, are the cause of death in the majority of mice injected with plutonium at the level of 2–3 $\mu\text{Ci/kg}$. The reduction of lifetime incidence of bone tumors following reduction of the skeletal burden of plutonium by chelation therapy with DTPA has been reported.⁽¹⁾ This reduction was proportional to the amount of skeletal plutonium removed in the case of plutonium administered in the polymeric form, but not in the case of monomeric plutonium. It was suggested that the monomeric form was a more effective carcinogenic agent. This work was based upon a relatively simple index of tumor incidence—the proportion of mice that had developed a tumor by the time of death. No attempt was made to assay the number of bone tumors present in each mouse, or group of mice, or to study and evaluate subtle and perhaps more significant nonmalignant pathologic changes occurring in the continually irradiated bone. Microscopic slides of standardized skeletal areas of all mice receiving either polymeric or monomeric plutonium, without DTPA therapy, have been prepared for detailed studies of the carcinogenic process following both forms of plutonium. Control mice are being used for a histological comparison of normal aging effects in bone. These studies are now in progress in cooperation with H. Lisco of the New England Deaconess Hospital.

Effect of DTPA and Vitamin A on Removal of Plutonium from Weanling Rats.—Monomeric plutonium was administered to weanling rats aged 21–27 days. Plutonium deposition at 6 days in the liver, spleen, and the bone of these young, actively growing rats was comparable to that in the year-old adults studied earlier, and the effect of DTPA on removal of plutonium was also comparable.⁽⁶⁾ Although the average liver deposition of monomeric plutonium was the same in these young rats as in adults, there was considerable individual variation.

In an attempt to promote increased plutonium removal from bone beyond that produced by DTPA,

vitamin A in doses sufficient to disturb bone metabolism and remodelling was administered by gavage to 21-day-old rats that had received monomeric plutonium 3 days previously. In controls, initial liver deposition of plutonium was unusually low and bone deposition unusually high in comparison to the values noted in the preceding experiment. Therapy with DTPA alone reduced skeletal content by 65%—a greater reduction than the 45–50% observed in adults and in the young rats of the preceding experiment. Administration of both vitamin A and DTPA reduced the skeletal plutonium by 73%, or almost to $\frac{1}{4}$ of the control level. The dosage schedule of the vitamin A retarded growth and caused marked skeletal changes, evident both on the roentgenogram and in the high incidence of breakage of long bones. Thus, although this experiment has demonstrated the ability of vitamin A-induced changes in bone metabolism to enhance significantly the removal of plutonium from the bone by chelation therapy, the practical usefulness of vitamin A is limited by the severity of its physiological effects.

Modification of DTPA to Improve Cellular Uptake.—One of the limitations of DTPA as a therapeutic agent is the intracellular exclusion of this small molecule, whereas polymeric plutonium is presumed to be in part intracellular. Work is, therefore, proceeding on the preparation of EDTA and DTPA polymers having molecular and physical properties conducive to phagocytic uptake, on the assumption that this will promote intracellular contact between the chelating agent and plutonium particulates.

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EFFECTS OF EXPERIMENTAL ALTERATION OF PHAGOCYTIC ACTIVITY ON TISSUE UPTAKE AND RETENTION OF POLYMERIC PLUTONIUM

Marcia W. Rosenthal

PURPOSE AND METHODS

The role of the reticuloendothelial system (RES), which consists of the phagocytic cells in the liver, spleen, bone marrow, and other tissues, in the initial uptake and in the retention of toxic radiocolloids in the body is almost unexplored. The present work was undertaken to increase our understanding of the mechanisms of deposition of polymeric plutonium, the ultimate goal being the reduction of its long-term retention in liver and bone marrow. The distribution of polymeric plutonium in bone and the RES tissues has been determined in mice when the plutonium was given after stimulation or depression of the RES or when it had been deposited in the tissues before RES stimulation.

The RES was stimulated by the administration of glucan, a highly purified neutral polysaccharide fraction of zymozan, causing increased phagocytic activity and increased liver and spleen weights.⁽¹⁾ These effects are probably brought about through promotion of increased numbers of phagocytic cells, since administration of zymozan has been shown to cause marked proliferation of hepatic littoral cells, but not of parenchymal cells.⁽²⁾ Depression of the RES was achieved by either the carbon block technique or by administration of methyl palmitate. The mechanism of carbon block was originally described as a saturation of the phagocytic cells by the carbon particles,⁽³⁾ but it is now recognized to be quite complex.⁽⁴⁻⁶⁾ Methyl palmitate, apparently not phagocytized itself and quickly eliminated from the body, severely depresses the phagocytic activity of the Kupffer cells in the liver without altering the functional activity of the parenchymal cells.^(7, 8) Unlike some simple lipid complexes which depress RES activity, it causes no obvious histological damage.⁽⁷⁾

The functional state of the RES was assayed by determination of the disappearance rate from the blood of a standardized preparation of colloidal carbon after intravenous injection.⁽³⁾

A colloidal form of plutonium (30% ultrafilterable), 0.1 μ Ci per mouse, was administered intravenously to 10½-week-old CF 1/ANL male mice at a time when the RES was optimally stimulated with glucan (at 9 days) or depressed with carbon or methyl palmitate (at 5 hours and 1 day, respectively). Treatment schedules and doses of glucan and of methyl palmitate (suspended in Tween 20 and homogenized for 10 minutes in a Virtis homogenizer) were comparable to

those used earlier;^(1, 7) 160 mg/kg of colloidal carbon was used for the carbon block. These mice were killed 5 days after plutonium administration. Additional mice which received glucan 5 days after administration of plutonium, when the radioelement was deposited in the tissues, were killed 16 or 49 days after plutonium (9 or 42 days after the last injection of glucan).

PROGRESS REPORT

We have confirmed earlier work⁽¹⁾ which showed that glucan causes a peak of RES stimulation in mice 10 days after termination of injections (hepatosplenomegaly and optimal phagocytic activity as measured by the carbon technique). We failed to observe, however, the subsequent RES depression at 25 days with a return to normal by 30 days noted by these authors. In our experiment, spleen weights were significantly elevated for at least 42 days, up to 250% of the values in saline controls. Liver weights were increased significantly for 15 days (from about 20% to 75% of the control weights) and to a lesser extent for at least 42 days. The mean disappearance rate of carbon from the blood had returned to normal by 38 days, although some individual mice were still stimulated by 42 days. In addition to the peak of RES activity at 9 days, there was a second increase at 33 days evidenced by increased blood carbon clearance rate and increased spleen weight, but no increase in liver weight. Histological studies of livers and spleens from representative mice at all eleven time intervals of this experiment are in progress.

Effect of Depression or Stimulation of the RES Prior to Plutonium Injection

At the time of injection of plutonium into RES-stimulated or depressed mice, the functional state of the RES was determined in additional treated mice and expressed as the half-time ($t/2$) of disappearance of the test dose of carbon from the circulating blood. In saline groups and in Tween 20 controls (for methyl palmitate only) it was 21.0 ± 0.8 and 13.6 ± 1.6 minutes, respectively. In carbon-blocked, methyl palmitate-depressed, and glucan-stimulated groups the $t/2$ was 58.5 ± 3.6 , 95.8 ± 12.7 , and 11.3 ± 1.8 minutes, respectively. The rate of disappearance of the injected plutonium was not determined.

Five days after the injection of plutonium, the liver contained the same amount of plutonium (about one-

half of the injected dose) in saline and Tween 20 controls and in both groups of RES-depressed mice. Mice previously treated with methyl palmitate had 30% more plutonium in the bone and 15% less in the marrow than the controls. Mice pretreated with colloidal carbon showed no significant change in the amount of plutonium in the bone, but had 50% and 26% more plutonium than the controls in the spleen and bone marrow, respectively. Mice in which the RES had been stimulated with glucan prior to plutonium administration showed no differences from controls in the plutonium content of liver, spleen, bone marrow, or bone at 5 days.

Effect of Glucan Stimulation after Deposition of Plutonium

Sixteen and 49 days after plutonium injection the saline control mice showed a progressive loss of plutonium from the liver, but no significant change in plutonium content of spleen, bone, or bone marrow. In glucan-treated mice at 16 days after plutonium, when RES stimulation by glucan was at its 9-day peak, the liver had not lost quite as much plutonium as the controls, but the plutonium content of all other organs was unchanged. By 49 days after plutonium, however, when glucan stimulation of the RES was nearing termination, redistribution of the deposited plutonium was evident in the stimulated group. The liver content of plutonium was significantly lower than in controls and the skeletal content was higher by about the same amount (8% of the injected dose), computing total skeletal burden as the amount in both femurs $\times 12.5$.⁽⁹⁾ No change from control values was observed in the amount of plutonium in the marrow or the spleen.

CONCLUSIONS

Neither stimulation nor depression of the RES prior to administration of polymeric plutonium had any effect on the deposition of plutonium in the liver 5 days later. In the RES-depressed groups some changes were noted in the distribution of plutonium in other tissues: increased amount in bone, less in marrow following methyl palmitate; higher concentrations in spleen and marrow following carbon block. These changes suggest that the rate of blood disappearance of plutonium may have been slowed by the RES depression, thereby permitting the observed changes in plutonium uptake in bone, spleen, and marrow, although final deposition in liver was not altered.

Possible explanations for the lack of effect of functional alteration of the phagocytic activity on deposition of colloidal plutonium in liver, the major RES organ, may be offered: a) the effect on the RES may not have been of sufficient duration; b) the rate of

disappearance of plutonium from the blood or its rate of uptake in the liver may not be correlated with the final amount of hepatic deposition; c) the effect of the compounds employed may be relatively specific, i.e., the effect on the behavior of one colloid, carbon, may be different from the effect on another, plutonium. It is obvious that the final explanation will require a better understanding of the mechanisms involved in carbon and methyl palmitate depression, the specificity of RES blockade, the duration of methyl palmitate depression, and the rates of blood disappearance and liver uptake of plutonium under comparable conditions. However, none of the explanations listed above is completely satisfactory, especially in the case of glucan. The glucan stimulation of the RES certainly lasted until the sacrifice of these animals, and other evidence indicates that its effect on the RES can be observed with a number of different colloids and particulates. A further possibility is that the phagocytic cells of the liver do not play as important a role in the initial uptake of colloidal plutonium as originally proposed,⁽¹⁰⁾ and that the hepatic parenchymal cells, which were presumably not affected by the three agents used here, may be involved in the hepatic uptake of plutonium.

Glucan treatment initiated 5 days after injection of polymeric plutonium was followed by a decrease in the plutonium content of the liver. This effect occurred after the peak of glucan stimulation and was measured when the influence of glucan on the liver was probably over. In untreated animals, or in those receiving chelation therapy, the plutonium lost from the liver is not appreciably translocated to the bone and is excreted.⁽¹⁰⁾ In this experiment, however, the additional plutonium lost from the liver appears to have been relocated in bone. This depletion of colloidal plutonium in the liver by glucan stimulation of the RES, if confirmed, may be useful in combination with chelation therapy which should prevent translocation to bone and promote excretion.

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BIOCHEMISTRY OF PREOSSEOUS CARTILAGE

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PURPOSE AND METHODS

This study is designed to investigate the biochemical bases for the transformation of cartilage to bone. The over-all objectives are to increase our understanding of normal and abnormal physiological processes in bone and to develop new concepts and new techniques for the treatment of radioelement poisoning. The approach includes: 1) characterization of components of the ossifying cartilage and bone, particularly the mucopolysaccharide-containing components as they exist *in situ*; and 2) study of their interactions with other substances such as calcium and the polyvalent radioelements taken up by bone.

Current efforts are directed toward isolation, characterization and quantitation of undegraded components of the organic matrix of cartilage and bone at various stages during calcification and ossification. Of particular interest are the properties of complexes of mucoproteins, lipo- and phosphoproteins, and of associated enzymatic control mechanisms. We are also looking for changes in the physical and chemical properties of the undegraded organic and inorganic components of ossifying cartilage to determine their possible involvement in the uptake of calcium and other substances. Techniques for isolation and characterization of cartilage components have been described earlier.⁽¹⁾

A great obstacle to understanding the role of connective tissue components in calcification and ossification is the scarcity of reliable methodology. Therefore, the development of new or better methods for characterizing the organic components of cartilage is a continuing secondary objective in this work.

PROGRESS REPORT

The present report describes results obtained with calf scapula cartilage. Selection of this tissue is based upon the ease of obtaining ample quantity of fresh mammalian cartilage in any desired stage of transformation into bone. Following is a summary of current results obtained with cartilage of the ossifying zone (the 2-mm-wide region containing enlarged columnar cells which is about to be replaced by primary bone) and from the resting zone (the cartilage distal to the ossifying zone).

Swelling

In ossifying cartilage the content of water that is easily removed by air drying is about 80%, as compared with about 60% in resting cartilage. Strips of ossifying cartilage dehydrate more rapidly, and take up water much more rapidly and extensively after dehydration, than resting cartilage strips. Swelling was found to be pH dependent, with a large peak around 5.3 for fresh ossifying cartilage, but with almost no peak for resting cartilage.

Release of Mucopolysaccharide-Containing Components

Concomitant with swelling there is a release of acid mucopolysaccharide-containing material, with maximum release occurring at pH 4.2. While chondroitin sulfate-protein complexes have been shown previously to be readily water-extractable from homogenized cartilage, among other substances found to be spontaneously released from fresh intact cartilage strips on incubation in pH-adjusted water at 37° were inorganic orthophosphate and sialic acid. The latter has been

shown to occur mainly as sialoprotein. Again, much more was released from fresh or dried ossifying cartilage than from resting cartilage, with strong temperature dependence in both zones between 20° and 37°. (2) In contrast, after incubating strips of newly formed bone in water or buffer solutions, no sialic acid could be detected. The sialoproteins extracted from ossifying cartilage have been demonstrated, by moving boundary electrophoresis, to be heterogeneous in nature, as found previously for extracted chondroitin sulfate-protein complexes.

Phosphatase and Phosphate Release

The spontaneous release of orthophosphate from fresh intact cartilage strips noted above was also found to be pH dependent; there were significant increases in the amounts of inorganic phosphate released from ossifying strips at both high and low pH regions (14 μ M and 15 μ M P_i per gram per 3 hours' incubation at 37° from resting cartilage at pH 4.0 and 8.0, respectively, *vs.* 58 μ M and 65 μ M released from ossifying cartilage at pH 4.0 and 8.0, respectively). The presence of phosphatase, as indicated by the splitting of β -glycerophosphate and *p*-nitrophenyl phosphate, and a large rise in enzymatic activity in the ossifying zone, is shown in Table 73.

Stimulated by previous demonstration of pyrophosphate and pyrophosphatase in ossifying tissue, (3, 4) some preliminary work on pyrophosphatase activity in the two zones of calf scapula has been done (Table 74). Table 74 also shows some newly obtained results, as yet not well understood, indicating either an apparent inhibition of pyrophosphatase activity by barbital buffer or its activation by tris(hydroxymethyl)-aminomethane (Tris). Whatever the explanation may be, it was found, for intact cartilage strips of either zone incubated with sodium pyrophosphate, that upon

TABLE 74. PYROPHOSPHATASE ACTIVITY OF INTACT FRESH CARTILAGE*

Treatment	μ M P_i /gram cartilage			
	Resting		Ossifying	
	with P-P	no P-P	with P-P	no P-P
0.1 M Acetate buffer, pH 4.4, 24 hr	38.4	15.4	380	87.0
0.1 M Acetate buffer, pH 5.2, 24 hr	52.7	18.0	374	46.5
0.1 M Barbital buffer, pH 7.0, 24 hr	25.0	14.8	150	10.5
0.1 M Tris buffer, pH 7.0, 2 hr	—	30.9	—	35.3
0.05 M Tris buffer, pH 7.0, 2 hr, ‡ (following pretreatment in barbital at pH 7.0 for 24 hr and water wash)	118	—	366	—
0.1 M Barbital buffer, pH 8.0, 24 hr	15.2	16.4	104	14.5
0.1 M Tris buffer, pH 8.0, 2 hr ‡	—	19.7	—	59.0
0.5 M Tris buffer, pH 8.0, 2 hr ‡ (following pretreatment in barbital at pH 8.0 for 24 hr and water wash)	98.2	—	289	—

* About 500 mg resting cartilage or 100 mg ossifying cartilage incubated at 37° with and without 10 μ M sodium pyrophosphate (P-P) in 5 ml of medium.

‡ No appreciable increase in P_i on longer incubation.

replacement of barbital buffer by Tris buffer at pH 7.0 and 8.0 there was a large increase in the amount of inorganic orthophosphate produced. As also indicated in Table 74, incubation in Tris at pH 7–8 without prior treatment or addition of pyrophosphate resulted in increased production of inorganic orthophosphate.

Analysis of Mucopolysaccharides and Mucoproteins

The work summarized here is the continuation of a collaborative effort between one of us (A. L.) and Dr. K. E. Kuettner, a former ANL Postdoctoral Fellow, involving the use of an automatic titration technique for the rapid determination of the equivalent weights and the sulfate and carboxyl contents of heparinic acid preparations. (5) This technique, as performed in aqueous solutions of chondroitin sulfuric acids, was found to give poorly defined sulfate inflections, resulting in difficulties in the assay of sulfate. Performing such titrations in partially aqueous solutions, composed of 70–80% dioxane in water, markedly enhanced the sulfate inflection of chondroitin sulfate preparations. (6) In addition, partially aqueous titration systems have now been found to be useful for the characterization of both acid mucopolysaccharides and the complexes of these substances obtained from preosseous cartilage.

TABLE 73. PHOSPHATASE ACTIVITY OF CALF SCAPULA CARTILAGE HOMOGENATES

Substrate	μ M P_i /mg protein/hr at 37°	
	Resting	Ossifying
β -glycerophosphate		
pH 5.8	1.24	3.4
pH 10.0	1.66	17.8
<i>p</i> -nitrophenyl phosphate		
pH 5.0	0.105	5.92
pH 5.0*	0.500	9.8
pH 10.0	0.08	11.2
pH 10.0*	0.225	25.0

* Supernate after centrifugation.

CONCLUSIONS

Following the initial investigation of the mucoproteins of resting and rapidly transforming ("ossifying") zones of preosseous cartilage, further work has indicated numerous other differences in the composition and functions of these zones. The calf scapula continues to be a valuable tissue on which to carry out this work. The results on swelling and release of mucopolysaccharide-containing materials bring us to a closer understanding of the function of swelling, which may be to raise the local concentration of metabolites (including calcium and phosphate), and of the possible control by mucoproteins and allied constituents of the deposition of inorganic ions on the surfaces of the cartilage matrix. The most recent efforts indicate the need for looking further into changes in enzymatic constituents, the presence and character of phospholipids, and the over-all functions of these substances in the

early biochemical transformations leading to calcium uptake and ossification.

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EFFECT OF NITROGEN MUSTARD ON THE RADIORESTORATIVE PORTION OF AMOEBA PROTOPLASM

Edward W. Daniels

PURPOSE AND METHODS

The primary purpose of this research is to learn more about the characteristics of the radiotherapeutic components in nonirradiated amoeba (*Pelomyxa illinoisensis*) protoplasm. It also is designed to yield information about the irreversible site of lethal radiation damage in the cell. The term irreversible is used here in relation to the irradiated cell itself, and it implies only that the exposed cell without therapy, cannot repair the radiation damage which it has received during a lethal exposure.

In general, the approach to this problem has been to methodically eliminate or inactivate specific systems in the donor protoplasm by the use of microsurgery, chemical compounds, or physical agents, to see what components are essential for radiorestitution. Previous work^(1, 2) has shown that protoplasm from supra-lethally nitrogen-mustard-treated* amoebae is, like nontreated protoplasm, a potent radiorestorative agent.

The method in the present work involved the immersion of the amoebae into fresh buffered solutions of nitrogen mustard for a period of 1 hour. They were

then washed in three complete changes of buffer,⁽³⁾ and cultured with food organisms (paramecia and chilomonas). Isolate and mass cultures were made of both control and experimental amoebae.

As a routine procedure, portions cut from lethally HN2-treated donor amoebae were injected by microfusion into whole, recipient amoebae which had previously received 30 kr of x-rays. This is slightly more than twice the minimum lethal dose.⁽⁴⁾ The microtransfer was done over a period of about 30 hours after HN2-treatment of donors and x-ray exposure of the recipients. Each transfer required about 30 minutes.

PROGRESS REPORT

In the past year, progress has been made in establishing the lower limits of sensitivity of the amoebae to HN2. It was also shown that the nonirradiated donor amoebae can receive at least 4 times the lethal dose of nitrogen mustard and still be capable of reversing the effect of more than twice the lethal dose of x-radiation in recipient amoebae (Figures 100-102). Lethal doses of HN2 ranged from 0.05 mg/ml upward, as shown in Figure 100. Cell division did not occur in any of the cells which received doses of HN2 ⁻¹ or above the lethal concentration level. However amoebae that received the lower lethal doses lived significantly longer (Figure 101) than those that

* Mustargen: Mustargen hydrochloride; mechlorethamine hydrochloride; HN2; methyl-bis(β -chloroethyl)amine hydrochloride; nitrogen mustard. The drug was obtained from Merck Sharp and Dohme, Division of Merck and Co. Inc., Philadelphia, Pa.

received higher doses (Figure 102). This phenomenon of lifetime shortening in moribund cells does not occur following x-irradiation, at least in the dose range between 1 and 4 times the lethal exposure level. On the other hand, lifetime shortening of lethally ultraviolet-irradiated *P. illinoisensis* amoebae is observed as the dose is increased above the lethal level into the supra-lethal range.

CONCLUSIONS

This work clearly shows that the site of lethal HN2 damage within the cell is different from the site of damage induced by x-radiation. Nonirradiated cytoplasm (without nuclei) can completely restore supra-lethally x-irradiated amoebae.⁽¹⁾ Thus, we know that the nuclei in the exposed cells recover, and that new nuclei are not needed for therapy if nonirradiated cytoplasm is injected into the irradiated cells. The irreversible site of lethal x-ray damage appears to be intracytoplasmic, rather than intranuclear. On the other hand, the nitrogen mustards react primarily with DNA, at the N-7 position of guanine, and the RNA to a lesser degree.⁽⁵⁾ Death in nitrogen-mustard-treated cells (Figures 101 and 102) apparently results from nuclear damage. The differential action of nitrogen mustard on intracellular components other than DNA is poorly understood. However, as shown by the work presented here, none of these reactions sufficiently damages the therapeutic components of unirradiated cells to reduce their radiorestorative value significantly.

Specific components in nonirradiated cytoplasm

FIG. 100.—Effect of nitrogen mustard (HN2) on the radio-sensitive amoeba, *Pelomyxa illinoisensis*.

FIG. 101.—Cell division in supralethally x-irradiated amoebae after therapy (Experiment A). N_0 , number of starting clones; N , number of clones surviving; N_t , total number of amoebae at time t .

FIG. 102.—Cell division in supralethally x-irradiated amoebae after therapy (Experiment B). N_0 , number of starting clones; N , number of clones surviving; N_t , total number of amoebae at time t .

which might have restorative value, principally the mitochondria, groundplasm, and the ribosomes, have been discussed previously.⁽⁴⁾ If messenger RNA in the donor protoplasm escapes significant HN2 damage, it might repair DNA in the nuclei of lethally x-irradiated recipients. Some groundplasm, which presumably contains glycolytic enzymes, might be required for radio-restoration. Glycolysis is inhibited by nitrogen mustard

which readily alkylates hexokinase.⁽⁵⁾ However, this would probably not reduce the potency of donor groundplasm, if the groundplasm is required and if the critical site of radiation damage in x-irradiated recipient cells is not at the hexokinase site. The radio-restorative value of mitochondria is being studied. At the present time, no information in regard to the presence of DNA in the mitochondria of *Pelomyxa illinoisensis* amoebae is available. Presumably, if DNA were present in the mitochondria it would be alkylated by HN2 treatment.

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EXPLORATIONS IN THE PREPARATION OF HOMOGENATE MEDIA FOR AMOEBAE

Edward W. Daniels and Evelyn P. Breyer

PURPOSE AND METHODS

Conventional homogenate media have failed to maintain the radiotherapeutic capacity of amoeba protoplasm following the breakdown of the plasma-membrane. Only direct micurgical transfer of protoplasm or cytoplasm from unirradiated donor into lethally-irradiated recipient amoebae has permanently restored exposed cells. It is important, for forward progress in this research, to perfect a procedure for pooling the protoplasm of many unirradiated donor amoebae so that it may be more extensively fractionated into its various components. Consequently, a number of aqueous solutions were designed, prepared, and tested in attempts to produce a homogenate media that would satisfactorily preserve the radiorestorative component in protoplasm (Table 75).

The approach has been directed toward obtaining a medium with properties similar to the ground cytoplasm of the living amoebae. This includes the control of numerous parameters such as pH, osmotic pressure, electrolyte content, temperature, and time between homogenization and injection of the homogenate into irradiated cells. Previous work on amoeba homogenates has been reported.⁽¹⁻³⁾

Equipment and solutions of homogenate media were precooled overnight, and all homogenizations and injections were done in a cold room at -3°C . The amoebae were brought into the cold room immediately prior to use, washed twice in the experimental medium, then lightly centrifuged into a pellet. Amoebae from the pellet were placed on a glass slide, and excess medium was removed. A small piece of tinfoil $15\ \mu$

in thickness was placed on each side of the mass of amoebae to prevent crushing the nuclei. Then a second slide was placed over the amoebae, and they were crushed by pressing the slides together at the ends.

The glass-slide homogenizer with the fresh homogenate was then put in a phase microscopic field, and the protoplasm was examined. The cytoplasm was studied as well as the nuclei, but the latter proved to be the best indicators of damage. The homogenate preparations were observed until substantial damage was seen; this occurred between 1 minute and 1 hour, but usually within 10 to 15 minutes.

PROGRESS REPORT

The results of the tests of various solutions are summarized in Table 75.

CONCLUSIONS

Nuclei are extremely sensitive indicators of toxicity in freshly prepared homogenates, and the method presented here of using nuclei as indicators of toxicity is both time saving and informative. The perfect method of testing is that of injecting the experimental homogenate into lethally irradiated amoebae to ascertain its radiotherapeutic capabilities. However, this method is time consuming and tedious and, therefore, is reserved for only the most promising preparations.

A saturated aqueous solution of cholesterol best preserved the normal structure of the nuclei. Dextro of high molecular weight was also better than most other preparations which were tested. Cholesterol added to 1% dialyzed high molecular weight dextran

TABLE 75. HOMOGENATE MEDIA TESTED ON THE AMOEBA, *Pelomyxa Illinoisensis* IN 1965

Homogenate medium	pH	Concentration and results
Adenosine triphosphate (commercial)	6.5	Hydrogen ion conc. adjusted with NaOH. Conc. ca. 0.1 mg/ml. Nuclei immediately cleared in central area, but peripheral nucleoli remained in fragmented condition for more than 10 min.
Casein	6.7	Conc. solution. Nuclei in poor condition from the beginning. Considerable variability in nuclear size and condition. Nuclei look better after few min., as in proteose-peptone medium. Clusters of crystals observed in cytoplasm. Plasmalemma skins present in the homogenate.
Cholesterol (Prep. I)	7.2	Cholesterol, dissolved in diethyl ether, dropped into warm water making a saturated aqueous soln. This was cooled to -3°C filtered and aerated. Squash homogenates contained normal appearing nuclei for several min. (similar to nuclei in living cells). Then nucleoplasm showed clumping and clearing. Very little if any swelling occurred in nuclei.
Cholesterol in Langley salt soln. plus pyridoxine. (Prep. II)	7.0	Saline soln. (grams/liter of glass dist. water): K_2HPO_4 14 g; KII_2FO_4 5.4 g; NaCl 2.9 g; NaHCO_3 4.2 g; and MgSO_4 3.6 g. This soln. diluted 1:1 with glass dist. water and tested as a homogenate medium. Nuclei showed central clearing rather soon, but nucleoli were in good condition for more than 5 min. The salt soln. at full strength was saturated with cholesterol and cooled, then pyridoxine was added to make a 1% soln. Homogenate preps. with this medium were relatively poor with intranuclear clearing, some nuclear swelling, and nucleolar fragmentation.
Cholesterol (Prep. III)	ca.7	Concentrated soln. of cholesterol in diethyl ether dropped into warm (40°C) glass dist. water until the latter became cloudy. The ppt. was removed by filtration, and the soln. bubbled with air until ether fumes were no longer detected. The soln. was then boiled, cooled, aerated, and used as homogenate medium. The nuclei looked normal for 5 min. and contained nucleoli which appeared to be in perfect condition. Then central area of the nucleus gradually became agranular, and after 10 min. nuclei began to burst.
Cholesterol (Prep. IV)	ca.7	Concentrated soln. of cholesterol in chloroform dropped into warm glass dist. water. The soln. was filtered, boiled, and aerated twice for a total of 1 hour. No chloroform odor remained. Cooled to -3°C . Amoebae were washed twice in this soln. After homogenization the nuclei didn't last as long as in the cholesterol-ether prep. The nucleoli remained quite well, essentially normal, but the central area of the nucleus became lighter within 1 or 2 min. and the nuclei began to burst. About the same sequence of pathological changes occurred as in the cholesterol-ether preparation, but they appeared at a faster rate.
Cholesterol (Prep. V)	6.8	One part saturated aqueous soln. of cholesterol plus one part 4% Ficoll in glass distilled water (dialyzed 4 days against glass distilled water). Tested as a homogenate medium but results negative.
Cysteine	5.9	Concentrated soln. in K_2HPO_4 . The amoebae floated in this pilot test. This prep. diluted with glass dist. water until the amoebae sank. However, the nuclei showed immediate damage. The nucleoplasm became clumped in different areas and then faded.
Cysteine	7.2	A 1% soln. in K_2HPO_4 to bring the pH to 7.2. The amoebae floated in this also, but were washed in it, and skimmed from the top of the soln. for homogenate testing. However, the nuclei in the homogenate showed a rather rapid clearing of the chromatin area and also the nucleoli. Unsatisfactory.
Cytochrome c	7.25	Conc. in glass dist. water was about 1%. In the homogenate the nuclei were partially cleared at once, particularly in the central chromatin area. The nucleoli were visible as small dark granules. In peripheral areas of the nucleus there was some clumping of the nucleoplasmic granules. After several min. the nucleoli could still be seen, but the nuclei swelled somewhat and did not appear normal.
Dextran	6.8	Mol. wt. = 75,000. Not dialyzed. 1% aqueous soln. The nuclei looked good in the homogenate for the first several min., then began clearing as in glucose solns. but more slowly. Dialysis might substantially improve this.
Diacetin (glycerol-1,3-diacetate)	6.8	Amoeba buffer ⁽⁴⁾ used to make 0.5% soln. This conc. killed all amoebae, paramecia, and chilomonads which were cultured in it for four days.
Diacetin (glycerol-1,3-diacetate)	6.9	0.25% soln. of diacetin in amoeba buffer used as culture medium to test toxicity. This conc. killed all amoebae and ca. 50% of paramecia in 2 days. However, the chilomonads did not appear affected within this time period. Diacetin appeared to have a differential killing effect on these organisms.
Diacetin (glycerol-1,3-diacetate)	6.6	A 25% soln. made up in glass dist. water. This was tested as a homogenate medium. The nuclei appeared reasonably normal in granularity at first, but the nucleoplasm soon began to appear in clumps of moderated size. After about 10 min. many of the nuclei began to leak nucleoplasm. Some nuclei still looked good at this time but others had refractile centers. This 25% soln. is too dense to centrifuge the cells down into a single stratum.

TABLE 75. *Continued*

Homogenate medium	pH	Concentration and results
Diacetin (glycerol-1,3-diacetate)	6.9	10% aqueous soln. The cells stratified well. In the homogenate preps. the crystals were well preserved as in other diacetin solns. Nuclei do not look as well as they did in the 10% diacetin soln. with pH adjusted to 7.4. Granules in the nucleus stand out well as seen through the phase microscope. After 10 min. the nuclei showed nucleoplasmic clumping and light areas. However, the nucleoli were quite normal in appearance. The nuclei started to leak nucleoplasm, indicating nuclear envelope breakdown.
Diacetin (glycerol-1,3-diacetate)	7.4	10% aqueous soln. adjusted with NaOH. This soln. freezes (-3°C) when disturbed. As a homogenate medium, it is slightly better than other diacetate preps. tested. There are many normal appearing nuclei in the homogenate during the first few min., as compared with nuclei within living cells. After 10 min. in the homogenate prep., the cytoplasmic granules appeared normal but the nuclei began to leak nucleoplasm and deteriorate.
Diacetin (glycerol-1,3-diacetate)	7.0	5% aqueous soln. Nuclei in the homogenates appear better than those in the 10% soln. at pH 6.9; granular structure within the nucleus and the nucleoli look normal during the first several min. Then nuclei deteriorate.
Diacetin (glycerol-1,3-diacetate)	7.2	1% aqueous soln. The nuclei in the homogenate remained granular but clumping of the nucleoplasm occurred after a few min.
DNA	6.7	1% aqueous soln. of commercial DNA adjusted with NaOH. Homogenate nuclei show considerable variation (from good to bad) but in general, the fields examined looked quite poor. Many nuclei have cleared within a few min., thus losing their normal granular appearance. Other nuclei show clumping of granular structures within the central areas.
Fibrinogen (bovine)	6.6	A 1% aqueous soln. prepared and tested. The homogenate nuclei looked quite normal for 2 to 3 min., then some showed central clearing. However, after 15 min. the majority of the nuclei looked fairly well. About 1 in 50 had burst. The nucleoli looked good. This medium could be tested for radiorestorative capacity with reasonable expectancy.
Lecithin	ca.7	Saturated chloroform soln. of lecithin dropped into glass dist. water which had been heated to 40°C . The aqueous soln. was saturated, filtered, cooled, aerated, and used as homogenate medium. The homogenate nuclei were found to be in poor condition 1 min. after prep. Lecithin is far less effective than cholesterol in maintaining normal nuclear structure in homogenates.
Niacin	6.5	Adjusted with NaOH (saturated aqueous soln.). In homogenate trial the nucleoli remained normal in appearance for several min. However, the central areas of the nuclei cleared fairly soon (within a few min.).
Niacinamide	7.5	Saturated aqueous soln. diluted until amoebae sank in the centrifuge tube. The nuclei in the homogenate (except for the nucleoli) cleared almost at once, but the nucleoplasm was a lighter grey than that following ATP homogenization. The nucleoli appeared somewhat swollen and refractile but even after 10 min. they were clearly visible.
Orange juice	6.8	Juice from a fresh orange was dialyzed 2 days against glass distilled water, then neutralized with NaOH. In the homogenate test, the nuclei remained granular for about 10 min. The nucleoli remained normal in appearance for more than 15 min. under phase microscopic observation. About 5 min. after homogenization the central area of the nucleus began to clear and this became progressively worse. A few nuclei burst 10 min. after homogenization. Discarded.
Ovalbumin	6.7	Twice crystallized, 1 to 2% ovalbumin was adjusted to pH 6.7 with NaOH. In the homogenate test, the nuclei did not look well from the beginning, but they looked better than those in conventional sucrose homogenate media. Some granulation was present, as in normal nuclei, but there was some nuclear swelling. After 10 min., granular appearance still is present and the nucleoli can be seen, but the nuclei are swollen and many are broken up. The dark granules in the cytoplasm, probably the mitochondria, are missing in this prep. This is different from the picture seen in most of the other preps. reported in this table.
Proteose-peptone	6.9	A 2% aqueous soln. was prepared. In the squash homogenate the nuclei appeared irregularly shaped as if they were losing water. The inner areas of the nuclei also appeared abnormal, some having clumps of granules, and some being clear. Again, the dark cytoplasmic granules, probably mitochondria, were missing. After several min., the nuclei looked better than the average homogenate medium which was tested, but they were nevertheless abnormal.
Pyridoxine hydrochloride	7.0	A 1% aqueous soln. was made up and adjusted with NaOH. When homogenized in this prep. the amoeba nuclei were more granular and normal in appearance than they were following homogenization in most other vitamin media tested. However, the nucleoli did not stand out in the phase field as they did in other vitamin preps. Nuclei appear to have contracted slightly.

TABLE 75. *Continued*

Homogenate medium	pH	Concentration and results
Pyridoxine hydrochloride <i>cont.</i>	6.65	A 9% soln. in glass dist. water adjusted with NaOH. Nucleoli are much more distinct than they were in the 1% soln. However, the nuclei clear in their centers within 10 min. The 1% soln. appears slightly better than this one.
Pyruvic acid	6.65	A 1 to 20 dilution of pyruvic acid in water was made and adjusted with NaOH. The homogenate nuclei had good nucleoli but the central part of the nucleus cleared rather soon. The sodium salt of this acid would no doubt be better.
Riboflavin	6.65	Saturated aqueous soln. In the test homogenate the nucleoli in 25% of the nuclei looked normal even after several min., but the centers of the nuclei cleared. After about 10 min. the entire nucleus swelled somewhat and cleared soon afterwards. The riboflavin soln. froze more readily than water. Yellow crystals of the needle type (riboflavin) were seen in the homogenates.
Thiamine HCl	0.75	Saturated aqueous soln. diluted so that amoebae would settle in the centrifuge tube. pH adjusted with NaOH. After breaking amoebae in this medium, their nuclei cleared within the first few min. in the central area, but the peripheral nucleoli remained distinct.
"	6.9	A 1% aqueous soln. was adjusted with NaOH. The nuclei in the amoeba homogenate remained fairly normal with gradual central clearing after about 10 min. This prep. was nearly as good as the 1% pyridoxine soln.
Thiouracil	6.5	A saturated soln. was made up in phosphate buffer. The homogenate nuclei showed some fading immediately, but after several min. they were still quite granular, and the nucleoli were still quite distinct.
Thiourea	6.1	A saturated aqueous soln. (0.1 g/ml) was tested. The nuclei in the homogenate showed immediate detrimental effects with fading and intranuclear clumping.
Triacetin	6.8	A 1% aqueous soln. was a very poor homogenate medium. The nuclei cleared and began to swell within 2 to 3 min.

in water might be more effective than either of these media. Further experiments are now in progress using some of the information gained from the exploratory work reported here.

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RADIATION PROTECTION FOLLOWING ORAL ADMINISTRATION OF CERTAIN AMINOTHIOLS

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PURPOSE AND METHODS

In contrast to their striking efficiency as radioprotectors when given intravenously, neither cysteine nor 2-mercaptoethylamine has been reported to be particularly effective when administered orally.⁽¹⁻³⁾ In the course of exploring the radioprotective activities of certain aminothiols which were not soluble in water and were suspended in a solution of gum arabic before administration by stomach tube, cysteine and 2-mercaptoethylamine were dissolved in a solu-

tion of gum arabic and given similarly as control compounds. Contrary to expectation, the latter compounds proved to be markedly effective in protecting against the lethal effects of x-rays whereas the complexes of primary interest were not protective. Female CF $\times$ 1/Anl mice of ninety to one hundred days of age were used. These mice are free of the common murine bacterial pathogens and were supplied with sterilized water, bedding and cages. Sterile precautions were employed during the weighting, stomach intubation

FIG. 103.—Protective action of 2-mercaptoethylamine (MEA) administered orally. ●—●, HOH at one hour before 775 R; ○—○, MEA in HOH, 750 mg/kg body weight at one hour before 775 R; ×—×, MEA in gum arabic, 750 mg/kg body weight at one hour before 775 R. Ten CF#1/Anl mice per group. All solutions brought to pH 7–8 with 10 N NaOH.

and irradiation procedures. The mice were subjected to single, total-body exposures to 775 R of x-rays (250 kv, 15 ma, 0.5 mm Cu and 3.0 mm Bakelite filters, 30.5 cm target distance, 1.72 mm Cu half-value layer and 200 R/minute) at one hour following the administration of L-cysteine HCl (cysteine) and 2-mercaptoethylamine HCl (MEA) dissolved in a solution of gum arabic (1 g of gum arabic in 4 ml of distilled water) and neutralized. The solutions were made up so that a mouse weighing 30 g would receive a volume of 0.5 ml by a flexible polyethylene or a rigid steel stomach tube.

PROGRESS REPORT

Cysteine (1200 mg/kg of body weight) and MEA (150 mg/kg of body weight) given intravenously, immediately prior to exposure to 750 R, were followed

by a 100% survival at 30 days in contrast to 5% survival for the irradiated controls. A similar level of protection was found after the oral administration of MEA (750 mg/kg) dissolved in water or in a gum arabic solution (Figure 103). It was established that a linear relationship existed between the degree of protection and dosage of MEA over the range of 150 mg/kg to 850 mg/kg. The protection afforded by cysteine was of the same order as that described for MEA. Thus, 90% survival at 30 days may follow oral administration of cysteine in dosages of 2680 to 3000 mg/kg prior to 775 R which is 100% lethal for control mice in the same experiments.

Suggestions as to the locus of protection of the orally-administered aminothiols are found in an experiment employing a radiation dosage (1200 R) large enough to elicit death of mice in 5–8 days. Such early death is due primarily to damage of the intestinal mucosa. Cysteine or MEA treatment resulted in an increased survival time which suggests that the protective action may be exerted on the intestinal mucosa. Positive evidence for such local protection was found in a sequence of experiments by Latarjet *et al.*,⁽⁴⁾ using oral administration of 2-aminoethylisothiuronium. Experimental work on the protective action of these compounds given orally is being continued for the purpose of gaining further information on the nature and sites of their protective action as well as for getting quantitative data on their dose-reduction factors.

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THE INCORPORATION INTO CYTOPLASMIC ORGANELLES OF TRITIUM GIVEN IN THYMIDINE

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PURPOSE AND METHODS

Evidence that cytoplasmic organelles, including mitochondria,⁽¹⁻⁵⁾ plastids,⁽⁶⁻⁸⁾ and kinetosomes^(9, 10) contain DNA has led to a contemporary revival of the theory of plasmagenes, which at its extragenic limit pictures a cytoplasmic organelle as a quasiorganism symbiotic in its host cell and with a genome all its own. Such a view may be reinforced by, and partly overlaps the concept of episomes in microbial genetics. An alternative theory, appropriately termed "nucleo-centric" by David Nanney,⁽¹¹⁾ regards at its nucleogenic limit the nuclear chromosomes as the origin and source of all genetic (cistronic) information, including that which the cell translates into the form and substance of its own organelles. Proof of one theory or the other will depend chiefly upon experiments that measure 1) extent of hybridization between nuclear and cytoplasmic DNA's, 2) capacity of cytoplasmic DNA to replicate *in vivo* and *in vitro*, and 3) extent to which DNA is conserved or metabolized in the nucleus and in the cytoplasm. This report represents an incomplete but promising beginning to the third approach. We aim ultimately to discern, by means of kinetic studies of H³-labeling of organelles in cell lineages, whether certain organellar DNA's have their origins in the nucleus or in the cytoplasm.

Tritium Conservation in Cell Lineages Measured Directly with a Windowless Geiger Counter

Cells dividing at approximately two fissions per day were labeled for ~95 hours in lettuce tea medium containing tritiated thymidine (H³T) at a concentration of 375 μ Ci/ml (specific activity 13 Ci/mmoles; Schwarz Bioresearch). Following exposure to label, cells were washed thoroughly and placed in nine-spot depression plates—one cell per spot—containing bacterized lettuce tea medium. Of 95 isolates, 89 underwent division. At various time intervals following division, all but one of the daughter cells were removed with a micropipette, deposited on a coverslip and allowed to dry down. The remaining six isolates which

did not divide were sacrificed to obtain readings of label incorporation at f_0 . Sister cells were placed on the same coverslip though in separate drops. A thin carbon film was applied to the coverslips in a vacuum evaporator, and the coverslips were affixed to planchettes for insertion under the windowless Geiger detector (Beckman Lowbeta II). It was possible to obtain readings above the background of about 6–8 cpm through f_7 ; with detector backgrounds of 0.5 cpm, the limit will be at f_{10} or f_{11} . Figure 104 presents counts for nine randomly selected cell lines followed in the experiment.

PROGRESS REPORT

The results obtained conform to a pattern of conservation of label within each cell lineage which is in line with studies employing P³²⁽¹²⁾ as well as leucine,^(13, 14) and cytidine.⁽¹⁴⁾ Exponentially growing cells show an approximate halving of label at each successive generation. Deviation from a theoretical exponential decrease can be accounted for in assuming variation in initial incorporation of H³T per cell, an assumption supported by the range in counts per minute for cells sacrificed at f_0 . Furthermore, selected cell lines generally showed internal consistency (Figure 104). Self-absorption of tritium beta particles by cell cytoplasm is probably in part responsible for some of the variation; dried-down cells are infinitely thick with regard to the range of beta particles.^(14, 15) *In situ* treatment with either cold 5% trichloroacetic acid (TCA) or cold 1 N perchloric acid (PCA) of cells mounted on coverslips results in an increase in counts per minute over those obtained prior to extraction.

Present efforts with the windowless Geiger counter have emphasized an essentially quantitative approach to H³T incorporation and distribution in cell lineages. Forro has effectively employed such an approach (but another technique) in the study of chromosome replication in *Escherichia coli*.⁽¹⁶⁾ Applications of a qualitative nature are feasible, by suitable modification of experimental design, to distinguish between bound and unbound label, employing PCA, TCA, and nuclease

FIG. 104.—Semilogarithmic plot of counts per minute as a function of cell generation to demonstrate conservation of label in nine cell lineages. Data for f_0 represent counts obtained by sacrificing nondividers after labeling. Counts for an individual cell lineage are indicated on the graph by use of the same symbol for all generations. The solid line represents halving at successive generations of a cell line for which $f_0 = 1000$ cpm.

extractions, and its localization as indicated by light-microscope autoradiography.

Delay of One Generation in Thymidine Labeling of Trichocysts

Labeled trichocysts appear within half an hour after administration of a tritium-labeled amino acid such as leucine ($H^3\text{leu}$).^(13, 17) The percentage of trichocysts labeled in such a cell is a function of the age of the cell at which the pulse of labeled amino acid is given. The peak percentage of labeled trichocysts (about 15% labeled) occurs when the pulse is given at about the 8th or 10th hour in a clone with a 12-hour generation interval.⁽¹⁷⁾ Following a $H^3\text{leu}$ pulse of 10 minutes delivered at cell age four hours, between 1 and 5% of the trichocysts become labeled; the label is conserved for as many cell generations as are the labeled trichocysts, but the average percentage of trichocysts labeled per cell declines by approximately a factor of 2 each time a cell divides. In other words, the patterns of segregation of label are fully conservative at the organelle level (a once-labeled trichocyst remains fully labeled for its lifetime) but are approximately disper-

sive (with a distinct tendency towards semiconservative segregation^(13, 17) at the cellular level).

In striking contrast, following a short pulse of tritium-labeled thymidine given at cell age 4 hours (that point in the cell cycle at which DNA synthesis commences appreciably)⁽¹⁸⁾ no labeled trichocysts appear at all during the remainder of that fission interval (f_0). Shortly after cell division the f_1 fissions show no labeled trichocysts until about the fifth hour, after which time labeled trichocysts continue to appear in increasing numbers until by the next division (f_2) about half of all the trichocysts in the young f_2 fissions are labeled (Figure 105).

Assay Methods for DNA on Cell Organelles

Several meanings, not necessarily mutually exclusive, may be ascribed to the finding that a lag of one generation occurs before trichocysts are appreciably labeled (i.e., to an extent comparable within an order of magnitude to the degree of labeling caused by H^3 under otherwise comparable circumstances) by given in thymidine. It is, for example, possible that all, and probable that some of the isotope confined to

FIG. 105.—The relationship between time of application of a pulse of tritium-labeled leucine or thymidine ($H^3\text{leu}$, $H^3\text{T}$) and time of appearance of labeled trichocysts.

FIG. 106.—Diagrammatic representation of extraction and control techniques prior to electron microscopic autoradiography

trichocysts is no longer present in thymidine or in DNA. On the other hand, the removal of substantial quantities of H^3 by deoxyribonuclease (DNase) might suggest that at least some of the label had remained in thymidine and found its way into "trichocyst DNA." The assay method devised to distinguish between these possibilities is shown in Figure 106. The $H^3\text{T}$ -labeled cell in question is placed in a microdrop upon a carbon-coated grid on the autoradiographic grid holder previously described.⁽¹³⁾ A control cell that contains trichocysts labeled with $H^3\text{leu}$ is placed in another microdrop to attest to the absence of high levels of proteolytic activity in the nucleases employed. A third microdrop contains another control consisting of $H^3\text{T}$ - (or tritium-labeled uridine, $H^3\text{U}$) labeled cells of *E. coli*, strain 15 T-his-U- (S. S. Cohen) (Figure 106, post 1). Trichocysts are induced to discharge by electrocuting the *Paramecia* in their respective microdrops and all drops are dried down in a desiccator, after which the grids are exposed for several hours to formaldehyde. An appropriate nuclease or proteolytic enzyme is added to the samples on the grid posts

(Figure 106, post 2) and after incubation in a moist chamber at 37°C , the enzyme is removed by means of capillary action onto bibulous paper. The grids are then washed and dried, and nuclear emulsion (Ilford L-4) is applied by means previously described.⁽¹³⁾

Typical results are shown in Figures 107–115. In Figures 107–109 only H_2O was added. In parallel runs against $H^3\text{T}$ labeled bacteria in which cold 1 *N* perchloric acid was used, the results were essentially identical to that of Figure 107. In Figures 110–112, ribonuclease (RNase "5 $\times$ crystallized, protease-free," Sigma, at a concentration of 100 $\mu\text{g}/\text{ml}$ for 90 minutes) was used; in Figures 110–112 DNase ("electrophoretically purified, RNase free", Worthington, at a concentration of 100 $\mu\text{g}/\text{ml}$ for 90 minutes) was used. All cells were initially labeled with $H^3\text{T}$. In Figures 107, 110, and 111 no loss of label is evident from either water or from RNase extraction although the RNase-extracted bacteria are sometimes disfigured slightly (Figure 110). In contrast, DNase (Figure 113) is effective in removing nearly every grain from the $H^3\text{T}$ labeled bacteria. A time course for this reaction showed

FIGS. 107-115.—Electron micrographic autoradiographs of labeled and extracted bacteria and trichocysts; H₂O extraction, Figures 107-109; RNase extraction, Figures 110-112; DNase extraction, Figures 113-115. See also Table 76 and the text.

about 50% removal in five minutes, and nearly 90% removal in fifteen minutes. Parallel experiments with DNase *vs.* H³U-labeled bacteria, and with RNase *vs.* H³T-labeled bacteria have shown no significant extraction of the label, indicating at the same time the relatively high specificity of H³U for RNA labeling and of H³T for DNA labeling in *E. coli* 15 T⁻his⁻U⁻, as well as the purity of the nucleases employed. Table 76 summarizes the results of five separate experiments showing the action of RNase and DNase against trichocysts from cells labeled with H³T. Figures 108 and 114 are of trichocysts extracted with H₂O and DNase respectively, and are from Experiment No. 1 (Table 76). Figures 109, 112, and 115 represent H₂O, RNase and DNase extractions respectively from experiment No. 2 (Table 76). Here, in nearly every instance, in contrast to the bacterial results, the nucleases were about equally effective in removing some of the label. While the range is great between experiments, the extraction is significant in every case, whether in terms of percent of label extracted per trichocyst (30–80%) or in terms of percent of labeled trichocysts removed from the population (40–80%).

CONCLUSION

Tritium given in thymidine is conserved without significant loss within a given cell lineage for at least seven fission generations. The label is entirely absent from the cytoplasmic trichocysts for one generation following its administration; after this, considerable concentrations are found on trichocysts late in *f*₁ and early in *f*₂, at which time about half the trichocysts of the cell become newly labeled with H³ in a form extractable by either DNase or RNase. The trichocysts and their contained label are presumably conserved thereafter, consistent with the clonal conservation implied by the cell lineage, direct count data. In earlier studies of H³T labeling in *Paramecium*, Berech and van Wagendonk⁽¹⁹⁾ had reported that cytoplasmic labeling occurred that was not removable by hot acid hydrolysis, and discussed chromatographic evidence that uridylic acid from the RNA of such cells was radioactive. They concluded that cytoplasmic DNA (cDNA) was also present, and further that *Paramecium* converts thymidine into RNA precursors that then become incorporated into RNA. The present study in which the trichocyst label appears to represent RNA as well as cDNA, suggests that it and analogous cell organelles may constitute the cytoplasmic sites for the deposition as well as the conversion effect. Acid hydrolysis-stable cytoplasmic sites of H³T incorporation have also been demonstrated in *Tetrahymena*.⁽²⁰⁾ Whether H³T was incorporated

TABLE 76. ORGANELLES LABELED AT *f*₂ BY H³ GIVEN IN THYMIDINE AT *f*₀

Exp. No.	1	2	3	4	5	1	2	3	4	5
	Av. No. gns./labeled trichocyst					% Trichocysts labeled				
Extracted by										
H ₂ O	15.0	36.6	—	40.0	50.0	50.0	63.7	—	40.0	71.2
RNase	—	15.0	20.3	14.7	33.7	—	37.8	53.4	9.9	40.2
DNase	3.3	17.8	9.6	25.0	33.6	9.8	36.5	19.4	21.6	20.8
	Av. % of label extracted/trichocyst					% Labeled trichocysts removed				
H ₂ O	0	0	0	0	0	0	0	0	0	0
RNase	—	59	—	63	33	—	41	—	75	43
DNase	78	51	—	38	33	80	43	—	46	71

initially into pretrichocyst cDNA (or into protrichocyst, nuclear nDNA) remains an open question.

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TRICHOCYSTS OF THE CRYPTOMONAD, CYATHOMONAS TRUNCATA

Frederick L. Schuster

PURPOSE AND METHODS

The cryptomonads are a small group of protists within the Flagellata. Members of the group are characterized by the possession of trichocysts that differ

morphologically from ciliate organelles with the same name. Both classes of organelles are alike in that they are extruded by the organisms under a variety of conditions and that they underlie the pellicle or cell cortex

FIG. 116.—Oblique section through the anterior end of *Cyathomonas* showing a portion of the cup (C). The macrotrichocysts, eight of which are seen at this level, are organized roughly into two "arms" (Mt). Microtrichocysts (mt) are seen under the plasma membrane of the cell. Mitochondria (M) are also found under the plasma membrane. Kinetosomes can be recognized at (K). The gullet is seen in cross section at (G).

in orderly arrays. In *Cyathomonas truncata*, undischarged trichocysts are organized into a prominent band located at the anterior end of the organism, a feature that distinguishes this organism from other cryptomonads. This study began as an investigation of the ultrastructure of *Cyathomonas*, but recent efforts have been directed toward problems related to trichocyst structure, composition, and ontogeny.

Cultures of *Cyathomonas* were maintained in buffered beef-glucose medium bacterized with *Aero bacter aerogenes*. For electron microscopy, populations of cells were fixed in phosphate-buffered glutaraldehyde followed by a second fixation in Palade's fluid. Trichocyst discharge for whole-mount examination was effected by placing drops of fluid containing living cells on electron microscope grids and allowing the drops to dry down. Trichocysts are discharged during the drying process. Such whole-mount preparations were either shadowed with platinum-carbon or negatively-stained with phosphotungstic acid.

PROGRESS REPORT

The organism has an anterior, cup-shaped depression (*cyath* = cup) from which the flagella emerge and

FIG. 117.—Macrotrichocysts sectioned in various planes. The morphology exhibited by the trichocyst at the lower left is characteristic of a section through the center of the spool-shaped organelle. A faint layering may be seen in the lower trichocyst.

FIG. 118.—A discharged trichocyst of *Cyathomonas* shadowed with platinum-carbon. The shaft (S) is proximal; the tip (T) is distal. A small granule characteristically occurring at the distal end of the shaft is seen at (H). Inset shows negatively-stained trichocyst shaft. Stain is inside of the tubular structure. Granules can be seen along the inner wall of the tube. Both figures are at the same magnification.

beneath which is located the band of refractile granules (undischarged trichocysts). The general morphology of cryptomonad trichocysts has already been described.⁽¹⁻⁴⁾ There are about ten of these organelles located behind one another in an arrangement that, in transverse section (Figure 116), is horseshoe-shaped. Each trichocyst is located within a unit membrane-limited vesicle (Figure 117). The organelles in this band measure $0.4\text{--}0.6\ \mu$ in diameter and will be referred to as macrotrichocysts. A second class of trichocysts measuring about $0.2\ \mu$ in diameter and not readily visible at the light microscope level is found under the plasma membrane in about ten longitudinally-oriented rows. These will be referred to as microtrichocysts. Rows of microtrichocysts alternate with elongate mitochondria, contiguous with the plasma membrane (Figure 116). Both trichocyst types in the undischarged state show a lamination or period of about $5\text{ m}\mu$. Upon discharge, both macro- and microtrichocysts produce a structure consisting of a tubular shaft with a granule at the distal end, from which extends a pointed tip (Figure 118). The shaft produced, upon discharge of a macrotrichocyst, measures about $20\ \mu$

TABLE 77

	Macro-trichocyst	Micro-trichocyst	R body
<i>Undischarged</i>			
Diameter	0.4–0.6 μ	0.2 μ	0.54 μ
Layer thickness	5 m μ	5 m μ	13 m μ
<i>Discharged</i>			
Shaft length	20 μ	4 μ	8–20 μ
Shaft width (whole-mount)	0.1 μ	0.05 μ	0.5 μ
Shaft width (sections)	0.03–0.06 μ *	—	—
Wall thickness (sections)	4.3 m μ *	—	—

* It is not possible to distinguish between discharged macro- and microtrichocysts in sectioned material.

in length; the shaft resulting from discharge of a microtrichocyst measures about 4 μ .

An ultrastructural pattern, strikingly similar to that of the cryptomonad trichocysts, is seen in the refractile (R) bodies of kappa particles of *Paramecium*.⁽⁵⁾ Upon isolation and treatment with detergent, R bodies unwind producing a rigid ribbon of 8–20 μ in length and about 0.5 μ in width. Like the discharge of the trichocyst, the unwinding of R bodies is rapid. In its coiled state, the R body exhibits a width of about 13 m μ . Because of the morphological similarity of R bodies to trichocysts, it is possible to think of trichocyst discharge as the unwinding of a ribbon. In support of this concept, shafts of discharged macrotrichocysts are wider distally (about 120 m μ) than proximally (about 80 m μ). This inverse taper can be accounted for if a macrotrichocyst, as seen in Figure 117, began unwinding from the wider outer surface. Tapering is not evident in the shafts of microtrichocysts; this may be due to their more compact organiza-

tion. Another similarity between R bodies and trichocysts is the presence of 100 m μ granules in association with the shaft or ribbon of both structures. Table 77 summarizes data on both types of organelles.

Several unsuccessful attempts were made to establish *Cyathomonas* in bacteria-free culture media. Deprived of bacteria, the cells starved and began to dedifferentiate. The trichocysts, a more or less constant feature of *Cyathomonas*, were not evident in the cytoplasm of axenic cultures. Instead, food vacuoles of the starved cells were found to be filled with shafts of discharged trichocysts. This suggests that the trichocysts are a form of food reserve that the organism can discharge and then ingest or, carrying the analogy with R bodies still further, that they may be intracellular symbionts maintained only while the organism is well fed. As in many organisms which support intracellular symbionts, starvation may induce a termination of symbiosis when the host digests the symbiont.

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THE DEVELOPMENT OF GRANA IN THE PREPLASTIDS OF A CHLORELLA MUTANT AFTER PHOTOINDUCTION

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PURPOSE AND METHODS

Our purpose is to correlate development of lamellae with the synthesis of chlorophyll in the developing chloroplast. The *Chlorella* mutant C-10 in a stage-characterized population was used in these studies.⁽¹⁾ Cells from 72-hour cultures were exposed to various intensities and qualities of light with aliquots being taken at 0, 1, 2, 4, 8, 12, and 24 hours to determine the pigment production and ultrastructural changes. Meth-

ods employed include those previously described for chlorophyll assays and for electron microscopy.^(1, 2) In addition to these, the dip method of negative staining was used⁽³⁾ in which the cells were ruptured by means of pressure and shearing in a cell fractionator⁽⁴⁾ and then differentially centrifuged to isolate plastid fragments for negative staining with phosphotungstic acid. Finally, glutaraldehyde-osmium tetroxide fixation⁽⁵⁾ was used on intact cells to bring out the fine structure of the ground substance for study in thin sections.

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FIG. 119.—Section of a *Chlorella* preplastid at the start of induction, following growth in darkness. At this stage of development, the preplastid is found to contain the vesicular prolamellar bodies. KMnO_4 .

FIG. 121.—The lamellae here have formed definite pairs after 4 hours of illumination. KMnO_4 .

FIG. 120.—In the young chloroplast, after the second hour of illumination, single stranded lamellae are found with some pairing occurring. KMnO_4 .

FIG. 122.—In the mature chloroplast, stacking of lamellae is prominent. KMnO_4 .

FIGS. 123 and 124.—Chloroplasts treated with 10 $\mu\text{g/ml}$ of actinomycin D. Note the inhibited lamellar development, the predominance of single strands, and the large masses of the vesicular prolamellar body remaining. KMnO_4 .

FIG. 125.—Portion of a *Chlorella* chloroplast fixed by the glutaraldehyde-osmium tetroxide method. Ribosomes are clearly present in the cytoplasm (arrow). Within the chloroplast, particles which have the appearance of ribosomes are found (arrows). These chloroplast "ribosomes" are nearly half the size of the cytoplasmic ribosomes.

FIG. 126.—Electron micrograph showing the large and small discs of a single plastid revealed by pressure disruption and negative staining. In thin sections, such stacked discs appear as the stacked lamellae of Figures 122 and 125.

PROGRESS REPORT

The ultrastructural changes which one observes in plastid-like structures in cells fixed in KMnO_4 range from the vesicular prolamellar body of the preplastid⁽⁶⁾ to the many paired or stacked lamellae of the mature plastid. At the start of induction, following growth in darkness, preplastids are observed (Figure 119). After 1 hour of illumination, single stranded lamellae have formed and by the second hour, some pairing of lamellae has begun to occur (Figure 120). Definite and extensive pairs are found by the fourth hour (Figure 121), and finally, in the mature chloroplast, stacks of lamellae are prominent (Figure 122).

Figures 123 and 124 illustrate some of the changes observed in the plastids of cells after 16 hours of illumination ($43 \mu\text{W}/\text{cm}^2$ GE Red) in $10 \mu\text{g}/\text{ml}$ of actinomycin D. Lamellar development is strikingly inhibited, single strands predominate, and large masses of the vesicular prolamellar body remain. This inhibition of structural development correlates with a 20–30% reduction in chlorophyll synthesis.⁽¹⁾

Glutaraldehyde-osmium tetroxide fixation brings out the granular ground substance of the cell. Figure 125 illustrates particles present in the developing chloroplast which have the appearance of the chloroplast “ribosomes” described by Gunning⁽⁷⁾ in developing *Avena* plastids. Gunning finds these chloroplast “ribosomes” to be about two-thirds the size of the cytoplasmic ribosomes. Under our conditions, the chloroplast “ribosomes” range in size from 53A to 90A with a mean size of 63A, and the cytoplasmic ribosomes range in size from 145A to 180A with a mean size of 160A. These figures show that the chloroplast “ribosomes” of a *Chlorella* are only 40% the size of cytoplasmic ribosomes in the same cell.

In order to discern the full extent and form of whole intact lamellae within the developing plastids, the negative staining technique was employed. The negatively stained fragments (Figure 126) show discs and stacks of discs that we interpret as the single stranded lamellae of thin sections in the former case, and the double stranded lamellae in the latter.

When dark grown cells were exposed to blue light* (irradiance of $124 \mu\text{W}/\text{cm}^2$), a peculiar oscillation in the rate of chlorophyll production was observed. During the second hour of illumination, the rate of chlorophyll production was approximately half that of the first and third hour, and a reduced rate similar to that of the second hour frequently occurred during the fourth hour (Figure 127). Under these conditions, only paired lamellae were observed even after 24 hours' illumination.

* General Electric fluorescent blue lamp (F72T8-B).

FIG. 127.—The relationship between the rate of chlorophyll production and the time following induction. The open and closed circles represent duplicate samples. Illumination was continuous (except for 4-minute sampling intervals) at an irradiance of $124 \mu\text{W}/\text{cm}^2$ of blue light following induction (see text).

CONCLUSION

In stage-characterized populations of a chlorophyll-deficient mutant of *Chlorella*, the preplastid develops into a mature chloroplast following its induction by visible light. First, the vesicular prolamellar bodies disappear, and single stranded lamellae form. These are interpreted to represent single discs much like the disc-like units that constitute the grana of mature chloroplasts in higher plants.⁽⁸⁻¹⁰⁾ This step is correlated with an initial high rate of chlorophyll synthesis. It is followed by the production of paired lamellae (or discs), and this event initially correlates with reduced rates of chlorophyll synthesis. The developmental process within the plastid culminates in stacked lamellae (stacks of discs) and high rates of chlorophyll synthesis characteristic of the mature chloroplastic cell.

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FINE STRUCTURE OF THE HERBIVOROUS GIANT AMOEBA, *PELOMYXA PALUSTRIS*

Edward W. Daniels and Evelyn P. Breyer

PURPOSE AND METHODS

An electron microscope study of the algae-eating, giant amoeba, *Pelomyxa palustris* was made to explore its protoplasm, and to compare it with other amoebae. This amoeba requires very little if any oxygen. It is an academically interesting organism, potentially valuable for basic investigations on radiosensitivity, radio-restoration, intermediary metabolism, etc.

Kudo⁽¹⁾ reviewed the literature on *P. palustris* and reported new light-microscopic investigations. Until recently, efforts by several investigators to study these organisms with the electron microscope have failed; sand grains,* normally found in the protoplasm of this species, prevented thin sectioning (ca. 100 m μ) for electron microscopy. Andresen *et al.* in Denmark recently discovered *P. palustris*, free from sand grains, and have studied it with the electron microscope.⁽²⁾ Their work and ours⁽³⁾ were performed independently, without mutual awareness, until August, 1965.

P. palustris is difficult to culture in the laboratory; consequently, our amoebae were obtained directly from the natural habitat. Collections were made in an abandoned limestone quarry (4 miles southeast of Argonne National Laboratory) from time to time during the last 10 years.⁽¹⁾ Since the 4th of November, 1964, sand-free amoebae have been found in a new collection site about 50 meters from the place where sand-containing amoebae were collected. The new site is a small pond with a heavy deposition of leaves from a nearby cottonwood tree. The amoebae were processed

for electron microscopy as described previously.⁽⁴⁾ A total of 80 cells was studied.

PROGRESS REPORT

The plasmalemma of *P. palustris* is smooth, without the external fringe found on other giant amoebae. Its cytoplasm (Figures 128, 130 and 131, C) is relatively simple in fine structure and consists predominantly of a variety of small vesicles and larger (>1 μ) vacuoles; the largest are the food vacuoles (Figure 128, FV). Unlike the other giant amoebae and *Amoeba proteus*, no mitochondria, Golgi bodies, contractile vacuoles, or crystal vacuoles were observed in *P. palustris*. However, two types of cytoplasmic, rod-shaped bacteria were found. One type (Figure 128, B) occurred at random in the cytoplasm. The other was found both surrounding the nucleus and also freely distributed in the cytoplasm (Figures 128-131, Bn).

P. palustris is a multinucleate amoeba, and its most complex internal structures are the nuclei (Figures 128, 130 and 131, N). The nuclear envelope (Figure 129, NM) is double-walled and contains annuli similar in appearance to those of *P. illinoisensis*. Minute vesicles cover the external surface of the nuclear membrane (Figures 129 and 130, V). The peripheral bacteria (Figure 129, Bn) were separated from these vesicles by a clear, narrow region of cytoplasm. All nuclei were surrounded by bacteria. The nucleoli were located along the internal periphery of the nucleus (Figures 128 and 129, Nu), in addition to small nucleolar-like structures or micronucleoli (Figure 129, MNu). Nucleolar inclusions (Figure 129, NC), consisting of clusters of electron-opaque particulates, were frequently observed. Both the number of particulates in

* We have not done physical or chemical analysis for sand in *P. palustris*, but base our assumption on the reports of previous investigators⁽¹⁾ and on our inability to make thin sections of these amoebae with glass, quartz, or diamond knives.

FIG. 128.—A general view of *P. palustris* protoplasm showing nuclei (N) with nucleoli (Nu) and external peripheral bacteria (Bn). The cytoplasm (C) contains food vacuoles (FV) and another type of bacterium (B) as well as many vesicles and vacuoles.

a cluster, and the number of clusters was variable. However, this could have been the result of sectioning. The remainder of the nucleus contained granular nucleoplasm. No nuclear helices or filaments were seen.

Although nuclei were observed which differed from that just described, all of the nuclei within a given amoeba were similar in appearance, showing that they were synchronized in the same stage of the cell cycle. The former nucleus was seen most frequently. A brief description of those that differed and were seen less often follows.

The nuclei of some amoebae had inwardly serrated membranes (Figure 130, SM), as in *Amoeba proteus* nuclei at interphase.⁽⁵⁾ Extranucleolar clusters of elec-

tron-opaque particles were suspended in the nucleoplasm (Figure 130, Cl), and enlarged bacteria were present around the nucleus. Electron-opaque spheroids (about 0.1 μ), each within a loosely enveloping irregular vesicle, were found in the cytoplasm of amoebae which contained this type of nucleus (Figure 130, S).

Other amoebae had greatly enlarged nuclei with concentrations of what appeared to be chromatin material in their centers (Figure 131, Ch). However, we have not identified this material cytochemically. Electron-opaque fragments (Figure 131, Nu), possibly from nucleoli, surrounded this central material. Extranucleolar clusters of electron-opaque particles were observed within the central nuclear mass (Figure 131,

FIG. 129.—Enlargement of the type of nucleus seen in Figure 128 showing nucleoli (Nu), intranucleolar clusters (NC), micro-nucleoli (MNu), nuclear membrane (NM), minute vesicles (V) along this membrane, and peripheral bacteria (Bn).

FIG. 130.—Part of a nucleus (N) showing extranucleolar clusters (Cl) in the nucleoplasm, faint serrations (SM) along the inner surface of the nuclear membrane, and minute vesicles (V) covering the external surface. Also present, in the cytoplasm (C), are enlarged *nuclear bacteria* (Bn) and electron opaque spheroids (S).

Cl). Some of these clusters exhibited intricate geometrical arrangements. The rest of the nucleus contained homogeneous nucleoplasm. The nuclear envelope was double-walled with a lamellated band in place of the

minute external vesicles (Figure 131, L). Electr opaque, variable stellate structures (Figure 131, bu), were found in contact with the lamellated band and randomly distributed in the cytoplasm. The cytoplasm

FIG. 131.—A different nuclear stage showing enlarged nuclei (N), central concentrations of chromosome (Ch) and nucleolar-like (Nu) material, external lamellated band (L), and variable stellate structures (Bn). The cytoplasm (C) is typically quite vacuolated in this stage.

was particularly vacuolated in amoebae with this type of nucleus. Spherical to ovoid homogeneous aggregations, resembling vacuoles and roughly the size of these nuclei, were frequently seen in the cytoplasm. They were composed of numerous small rings of minute (ca. $20\text{ m}\mu$) particles. These aggregations were not enclosed in a vacuolar membrane, but were surrounded

by the variable stellate structures. Other than the nuclei, these aggregations were the only structures in the protoplasm that were encircled by these stellate bodies.

Amoebae also contained essentially spherical aggregations of homogeneous particles that had no stellate structures along their peripheries. Each of these

amoebae had one of two types of nuclei which were either 1) surrounded by a lamellated band and nearly empty vesicles, or 2) surrounded by minute irregular vesicles and enlarged peripheral bacteria. These irregular vesicles were not clearly separated from the cytoplasm. Furthermore, the nucleolar material in the latter type of nucleus was found either in a few large masses toward the periphery, or in a thin, fused peripheral layer. The homogeneous aggregations in amoebae with this type of nucleus were approximately 1.5 to 2 times the diameter of the nucleus.

CONCLUSIONS

Mitosis and division of *P. palustris* are not well understood. Our electron microscope evidence suggests that nuclei, such as those seen in Figures 130 and 131, are undergoing division and might be in prophase. The following transitions seem to occur during this stage: 1) the nucleolar clusters (Figure 129, NC) leave the nucleoli and migrate toward the inner surface of the nuclear membrane (Figure 130, CI); 2) the nucleoli then migrate toward the center of the nucleus and become fragmented around the chromatin that appears to be accumulating (Figure 131, Ch); and 3) the peripheral nucleolar clusters move inward after the nucleoli. However, no breakdown of the nuclear envelope was observed in any of the 80 amoebae which we studied nor was a cell or nucleus actually seen dividing. Nuclear division might occur without fragmentation of the nuclear envelope. Cell division, in which two similar daughters were produced from a single amoeba, was observed by one of us (E.W.D.) 7 or 8 years ago. This is in agreement with Kudo⁽¹⁾ who stated that these amoebae divide by plasmotomy into 2 or more daughters. Additional study is required to explain the ambiguities of division in this organism.

P. palustris lacks the Golgi bodies, mitochondria, and external plasmalemma fringe which are normally associated with the giant aerobic amoebae, but that

are typically absent in the parasitic amoebae, *Entamoeba histolytica*,^(6, 7) *E. invadens*, and *E. terrapinae*. These parasitic amoebae are either anaerobes or live in the presence of relatively low oxygen concentrations. *P. palustris* appears to live in an environment which is similarly low in oxygen. Its energy is probably obtained by way of the glycolytic pathway.

The homogeneous aggregations described in this report may consist of glycogen. Okada⁽⁸⁾ reported the presence of bacteria around *lucid bodies* that were present in large numbers in the cytoplasm of *P. palustris*; they were the size of nuclei, and they consisted of glycogen. The homogeneous aggregations answer this description and are the only structures other than nuclei around which bacteria-like forms collect. They are probably the same as the refringent bodies described by Kudo.⁽¹⁾

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STRUCTURAL DIFFERENCES WITHIN THE MITOCHONDRIA OF AMOEBAE DURING INANITION AND MITOSIS

Evelyn P. Breyer and Edward W. Daniels

PURPOSE AND METHODS

Pelomyxa carolinensis is a radioresistant species of amoeba, originally obtained from North Carolina. The presence of zig-zag tubules in the mitochondria of this amoeba was first demonstrated by Pappas,⁽¹⁾ Pappas and Brandt,⁽²⁾ and Borysko and Roslansky.⁽³⁾ In a study just completed,⁽⁴⁾ we found a definite correlation

between the early stages of mitosis and the formation of complex tubular patterns within the mitochondria of the radiosensitive amoeba, *Pelomyxa illinoisensis*. In this study, we wished to ascertain whether a relationship exists in *P. carolinensis* between intramitochondrial variations and changes in the cell cycle or food intake.

Well-fed, starved, and starved refed *P. carolinensis* amoebae of known mitotic stages were examined. The starvation periods varied from 3 to 6 days. Sixty amoebae were prepared for electron microscopy as previously described.⁽⁴⁾

PROGRESS REPORT

No mitochondria with complex tubular patterns were found in well-fed interphase or mitotic amoebae that previously had not been starved. Approximately 75% of the amoebae that were starved 5 to 6 days each had some mitochondria (ca. 20%) that contained complex internal patterns. About 75% of the amoebae that were fed for 1/2 to 1 hour after a 5-day fast had complex intramitochondrial patterns, but the percentage of mitochondria containing complex patterns within each organism was higher than in starved cells. New daughter cells, fasted 3 to 5 days, then continuously fed until they divided, contained no complex patterns.

A study* was also made on individual mitotic† amoebae that were selected from feeding cultures. However, the record of prior food intake for any single amoeba was unknown. About 35% of these amoebae contained some mitochondria (ca. 5%) with complex internal patterns. In this study, no complex patterns were seen in the interphase cells that were taken from the same culture.

CONCLUSIONS

Complex intramitochondrial patterns were found in starved *Pelomyxa carolinensis* amoebae. Variance of the starvation periods from 3 to 6 days did not affect these patterns. Refeeding starved amoebae for 1/2 to

1 hour appeared to increase the percentage per cell of mitochondria with complex patterns, but did not seem to affect the percentage of cells containing these patterns. However, feeding after rather severe starvation until mitosis occurred, completely eliminated the intramitochondrial complex patterns (a matter of several hours or more). Some complex intramitochondrial patterns were found in amoebae undergoing mitosis in well-fed mass cultures, but the individual nutritional history of each amoeba was unknown.

ATP may be synthesized at a faster rate in mitochondria which contain complex patterns as a result of an increased requirement for energy by the cell. In a previous paper,⁽⁴⁾ we postulated that complex patterns are formed in the mitochondria of the radiosensitive amoeba, *Pelomyxa illinoisensis* in response to a demand for more ATP at the start of mitosis. However, the radioresistant amoeba, *Pelomyxa carolinensis*, does not have complex intramitochondrial patterns during mitosis in continuously well-fed individuals. Furthermore, *P. illinoisensis* does not form complex intramitochondrial patterns during starvation, as does *P. carolinensis*. While we do not know the reasons for the occurrence of complex patterns at different times in the two species of amoebae, we have discovered another way in which radioresistant and radiosensitive amoebae differ.⁽⁴⁾

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FINE STRUCTURE OF THE MACRONUCLEUS AND MITOCHONDRIA IN ULTRACENTRIFUGED TETRAHYMENA CILIATES

Edward W. Daniels and Evelyn P. Breyer

PURPOSE AND METHODS

In our study of stratified amoeba nuclei,⁽¹⁾ we showed that the nucleoli migrate rapidly to the centrifugal pole of the nucleus. In *Amoeba proteus* the nucleoli stratified into two distinct layers. Because the *Tetrahymena* nucleus takes up H³-thymidine much more readily than the amoeba nucleus, we extended our

analysis of intranuclear structures to include the *Tetrahymena* ciliates. Our aim was to determine if tritiated-thymidine-labeled components of the nucleus could be separated from unlabeled moieties.

The *Tetrahymena* were from strain W, amiconucleate in character, and were grown axenically in 2% proteose peptone. The temperature was kept in the

* This work was done by Miss Doris Buer, Argonne ACM Semester Student, 1964.

† Mitotic: Selection was based on external morphology, and later checked by electron microscopy. However, there is still the possibility of some errors in the differentiation of prophase stages from interphase.

FIG. 132.—Centrifuged *Tetrahymena* showing stratification within the nucleus (N), two types of mitochondria (M and M₁) in the cytoplasm, and the cell wall (C).

FIG. 133.—Nucleus (N) of centrifuged *Tetrahymena* is shown stratified above a layer of mitochondria (M). Direction of centrifugal force is to the right.

range of 24 to 25°C. The labeling technique⁽²⁾ and the electron microscopic (EM) technique⁽³⁾ have been described.

PROGRESS REPORT

We were able to stratify the unlabeled ciliate nuclei as shown in Figures 132 and 133. A force of 140,000 *g* for 8 minutes at 15°C was most effective. Definite separation of at least two kinds of intranuclear electron-opaque components was obtained.

We found two kinds of mitochondria in our preparations (Figures 132 and 133, M and M₁). One of the two mitochondrial types (Figure 132, M₁) is expanded and appears to be more hydrated than the other, more contracted type (Figures 132 and 133, M). The latter is more electron-opaque.

Although not shown in the figures, fat globules in the *Tetrahymena* migrated to the centripetal pole of the cell. In a given EM section through the lipid cap, some 100 or more spherical globules are seen which measure from about 0.4 μ to some 1.4 μ in diameter. Large (ca. 5 μ), clear vacuoles, stratifying centrifugally to the lipid globules, were suspended in a finely granular cytoplasm. Below this, centrifugally, were food vacuoles, then the nucleus. Some of the mitochondria stratified at the level of the nucleus, but most of them were found centrifugal to the nucleus, indicating their greater density. The more compact type of mitochondria stratified centrifugally to the more hydrated type. Clear vacuoles, about the size of mitochondria, stratified at the level of, and centrifugally to the nucleus.

They were not stained, but they are probably starch, or a related polymer.

CONCLUSIONS

The two morphological types of mitochondria could represent two physiological states of a given species of mitochondria within a single cell. On the other hand, these differing mitochondria might be two separate and distinct types.

We have not yet stained or labeled the stratified nucleus, and can only speculate on the relative distribution of DNA, RNA, and protein. As judged from work on other cells,⁽⁴⁾ the migrating component at the centrifugal pole (Figures 132 and 133) is probably RNA and protein. We intend to label these ciliates with H³-thymidine, in one group of experiments, and H³-uridine in another group, and to utilize electron microscope autoradiography to distinguish the RNA from DNA in stratified nuclei.

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THE EFFECT OF FIXING AGENTS, CATIONS, ISOTONICITY AND pH ON CELLS PREPARED FOR ELECTRON MICROSCOPY

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PURPOSE AND METHODS

The present communication is an extension of previous reports.^(1, 2) Its purpose is to develop reliability and reproducibility in fixing the cellular components. Consistent reproducibility will enhance the determination of deviations produced experimentally. For this reason, a thorough investigation of the fixing properties of a number of agents combined with a number of salts and buffers is in progress.

The gonad of the male grasshopper (*Melanoplus ferentialis*) is the principal tissue used, although at times other tissues have also been used. The gonadal cell contains all the basic components seen in cells.

An understanding of the reaction of fixatives on tis-

sue organelles is necessary because the various cell components require specific relationships of the salts, buffers, and fixing agents for their individual enhancement. The proper isotonicity for the various organelles has previously been discussed. The necessary isotonicity was determined by the freezing point depression apparatus. All solutions were rendered normal saline equivalent (NSE) with respect to the ionic strength of the physiological saline for a specific cell or its components. The fixing solution was made NSE by the proper balance of fixative, salt or salts, and buffer. There follows a discussion of results obtained using various proportions of salt species and buffers. It was found that with specific fixing agents the amount of

FIG. 134.—Spermatocyte, fixed in glutaraldehyde buffered with phosphate, and post-fixed in KMnO_4 buffered with phosphate. The pH of both solutions was 6.7. Glutaraldehyde fixation, followed by KMnO_4 or OsO_4 , retains most of the cellular components. Nuclear contents (N), nuclear envelope (NE), cell and mitochondrial (M) matrices are preserved. Endoplasmic reticulum (ER), Golgi (G), and other membranes are very distinct. There is a tendency for some mitochondria to explode (arrow M), as mentioned in the text. Usually, vesicles at the Golgi ends also appear swollen (arrow G). Although chromatin and RNP granules can be seen with KMnO_4 post-fixation, these are more distinct with osmium post-fixation.

salt, the type of buffer, and the pH of the solution contributes to the final result obtained.

To study the effects of various salts on the tissue, all other constituents of the fixative were kept constant. To study the pH effect, a series of different buffers was used. A buffer that produced the pH desired was chosen. When several fixatives, i.e., glutaraldehyde, osmium tetroxide, or potassium permanganate were tested, the appropriate buffer for each fixative was used. Oxidation or reduction of the organic buffer by the fixing agent was prevented by using inorganic buffers in conjunction with strong oxidants. The buffers used in this study were phosphate, veronal, cacodylic acid, collidine, sodium borate, sodium acetate, and glycerine. These buffers provided a pH range from 1.2 to 9.

PROGRESS REPORT

Fixing Agents

Glutaraldehyde.—The several fixing agents studied produce specific cellular effects. Glutaraldehyde fixation is used when a specific protein, in the cell, is to be subjected to enzymolysis. This agent, an organic reductant, does not contain metal ions that reduce at the location of cell components. Because materials fixed in glutaraldehyde retain all organelles and most of their components, the cells appear well fixed but are low in contrast, and appear fogged. Cells fixed by it, must be post-fixed with a metal-containing agent to provide electron absorbers on the cell components and

FIG. 135.—Maturing spermatids, fixed in veronal-buffered OsO_4 made NSE with KCl. Osmium fixation employing KCl to achieve isotonicity yields excellent preservation of cellular contents. Shown are sperm middle piece with the "9+2" rod configuration (arrows) unusually well represented, and nine pairs of lighter staining filaments in the proximity of the rods. The microtubular complex (MT) surrounding the nebenkern (NK) and the tail filaments is clearly evident. In the cross section of the sperm head, this microtubular complex seen to encapsulate the condensed filamentous chromatin the nucleus (N). The periodicity of the cell membrane is not apparent with this fixation.

thus permit the production of useful electronmicrographs. Post-fixation of glutaraldehyde-fixed material is usually done with osmium tetroxide, an oxidant. Glutaraldehyde fixation may also be followed by application of potassium permanganate, resulting in the retention of nucleoproteins that are typically oxidized by permanganate alone. Glutaraldehyde causes distortion in some mitochondria. Portions of the cristae mitochondrialis become swollen. This suggests that mitochondrial oxidative enzymes break down some of the glutaraldehyde molecules. If this happens, intramitochondrial ionic strength would increase and endosmosis would follow. Mitochondrial swelling, under these circumstances, implies that the mitochondrial membrane is semipermeable. Swelling is not restricted to glutaraldehyde fixation; it also occurs following fixation with permanganate.⁽³⁾ Swelling, under the isotonic conditions associated with oxidative degradation by permanganate, is discussed later.

FIG. 136.—Spermatocyte, fixed in OsO_4 , with NaCl and buffered to pH 1.3 with $\text{Na}_2\text{HCit}/\text{HCl}$. The cell, fixed under isotonic conditions, shows membranes (NE, ER), filaments and chromatin retained. The matrices of the nucleus (N), cytoplasm and mitochondria (M) are washed out. The absence of the matrices suggests probable solation and displacement during dehydration. It is interesting to note that nuclear, mitochondrial and cell volumes are maintained under isotonic conditions.

FIG. 137.—Spermatocyte, fixed in OsO_4 , with NaCl and buffered to pH 9.9 with $\text{NaOH}/\text{NaH}_2\text{BO}_3$. Fixation at an alkaline pH produces an over-all lack of contrast. The majority of the characteristic membranous structures are missing. Both the nuclear (NE) and cell limits (CM) are poorly delineated although the intercellular space is distinct. The endoplasmic reticulum (ER) appears granular rather than filamentous. Mitochondrial cristae are lacking; only the profiles of the mitochondria (M) can be seen, suggesting that the outer membranes may be there. On the contrary, the Golgi (G), chromatin, and ground substance of all parts of the cell have been well preserved, and the RNP granules (arrows) are quite distinct.

Osmium Tetroxide.—Osmium tetroxide, first introduced by Palade⁽⁴⁾ for electron microscopy in 1952, is considered the best general fixing agent available. We agree with this opinion. Osmium is reduced by the cell components at the unsaturated bonds of organic molecules. The degradation of organic molecules by this oxidant is negligible. Osmium fixation results in the formation of a hard soap or an osmium proteinate, depending on the fixation of either fats or proteins. Cells fixed with this agent under NSE conditions do not swell or shrink and compare well with living cells examined with a phase-contrast microscope. Osmium-fixed material permits the identification of macromolecular arrangements of ribosomes, the endoplasmic re-

ticulum, mitochondrial elementary particles, chromatin strands, myofibrils and bands, cell membranes, the nuclear envelope, the Golgi apparatus, centrioles, and the tail filament bundles in sperm. Under high magnifications (80,000–160,000), periodicity that suggests macromolecular organization can be observed in several cell components, e.g., mitochondrial membranes and the spermatid cell membrane.

Potassium Permanganate.—Potassium permanganate is a stronger oxidizing agent than osmium tetroxide. Under low magnifications, cells fixed in permanganate appear excellent. At high magnifications, however, cell components appear granular. There appears to be a strong oxidative degeneration with this fixative that breaks down RNP (ribose nucleoprotein) granules completely, and the DNA (deoxyribonucleic acid) of the chromosomes appears to be leached out. Usually, centrioles are absent and myofibrils are lacking in such preparations. The elimination of these granules and organelles removes much of the fog, so that membranes of organelles are distinct. To study membrane topology at low magnifications, permanganate is preferred, but the presentation of the true ultrastructure of the membranes is impossible with this agent. Permanganate is a useful fixative if the experiment permits the elimination of interferences like those mentioned above.

Salts

Various salts were used to lower the freezing point of the fixatives (fixative-buffer mixture) to provide an ionic strength that is equivalent to normal saline. The salts used were NaCl, KCl, or CaCl_2 . The salt concentration was never equivalent to normal saline because the fixative contributed to part of the ionic strength in the solution. When phosphate-buffered osmium is supplemented with NaCl alone, the preparations show good over-all fixation. The addition of KCl alone, which is the normal intracellular salt, also renders good fixation. The RNP granules throughout the cytoplasm, as well as distinct dense areas of chromatin, are especially well retained with these salts. The structure of the nuclear envelope, however, is not clearly defined. If KCl is used with a combination of phosphate buffered osmium-permanganate fixative, intracellular membranes are retained. The above osmium-permanganate fixative with NaCl substitution for KCl does not fix intracellular membranes. CaCl_2 used with osmium produces a high granularity in the preparation, but periodicity in chromatin and the membranes is distinct. NaCl, KCl, and CaCl_2 used together at a ratio of 3:2:1 yield ideal general fixation of cells with osmium. Once again, the choice of the salt is important in order to enhance or suppress specific cellular struc-

tures. NH_4Cl yields very poor results when it is used with several fixing agents. Although this salt is used extensively for biochemical extractions and for salting-out at high concentrations, it is a very poor additive for the fixation of cells.

Buffers

Veronal Acetate.—Veronal acetate is the buffer used most frequently in producing electron microscopic preparations. Baker *et al.*⁽⁵⁾ have shown that its buffering effect is poor at pH 7.3, but the fixation of cells is very good. Tissues, when this buffer is used, are relatively small in volume in relation to the volume of the fixing solution. The maintenance of a pH of 7.3 with this buffer is important. Arsenate, veronal, cacodylic acid, collidine and some other buffers are used at a pH of 7.4 in combination with specific fixatives. These buffers, useful for maintaining specific hydrogen ion concentration of the normal cell, are foreign to it, physiologically.

Phosphate.—The buffering effect of phosphate is best at pH 6.8; at this pH, the curve for the buffer is midway along its plateau. It was used at this pH as the base line to compare other buffers at the same pH. At higher or lower pH, other buffers were used as standards.

pH

The pH effect is very important. We have found that even with isotonic conditions, protein and nucleoprotein isolation and gelation occurs. Between pH values of 1.2 and 4.0, cell membrane, nuclear envelope and chromatin are very compact. The cytoplasmic and the mitochondrial matrix as well as the nuclear matrix are washed out. NSE osmium-fixed material at pH values between 8.3 to 9.9 was compared with material fixed at the physiological range of pH 6.8 to 7.3. Cells fixed in the alkaline pH fixatives invariably lacked crispness and contrast found in the latter. Throughout the series the nuclear content and cytoplasmic ribonucleic acid particles were well preserved. Most cellular organelles of tissue fixed at pH 8.3 were missing or were barely discernable. At pH 9.9 membranes and mitochondria were present. This is indicative that the proteins, which have a specific isoelectric point, are affected by the pH of the fixing agent differentially. However, the better fixation at pH 9.9 than at pH 8.3 may be due to the atomic species of the different buffers used for different pH values. We cannot overlook this possibility, and this problem will be investigated more thoroughly.

Electron micrographs that were taken during 1 study are presented (Figures 134–137) together with captions indicating the salt-fixative and pH values used to produce specific effects identified.

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PREPARATION AND PROPERTIES OF PERMANGANIC ACID FOR POSSIBLE USE AS AN ELECTRON MICROSCOPY FIXATIVE

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PURPOSE AND METHODS

Aqueous KMnO_4 has been used successfully as an alternative to aqueous OsO_4 for electron microscopy fixation.⁽¹⁾ This prompted us to determine if an analog could be found that would similarly complement the vapor phase use of OsO_4 .⁽²⁾ Theoretically, only HMnO_4 seemed possible.

Previous attempts to prepare it yielded only a product grossly contaminated with MnO_2 ; the HMnO_4 proved very unstable at room temperature.⁽³⁾ We felt that it might be sufficiently stable at 1°C (an often used temperature in biological research) and yet sufficiently volatile⁽⁴⁾ to be useful as a vapor phase fixative. By analogy with HClO_4 , it was anticipated that HMnO_4 might be less volatile than one of its hydrates, e.g., $\text{HMnO}_4 \cdot 2\text{H}_2\text{O}$. Accordingly, we devised a low-temperature method of purification depending on fractional vacuum sublimation of a frozen aqueous HMnO_4 solution which promised to yield both anhydrous and hydrated HMnO_4 .

PROGRESS REPORT

Preparation.—Samples of $\text{Ba}(\text{MnO}_4)_2$ and H_2SO_4 were analyzed by the usual gravimetric and volumetric procedures⁽⁵⁾ to $\pm 0.1\%$. To 120 ml of 0.3 M $\text{Ba}(\text{MnO}_4)_2$, was added a precisely equivalent amount of H_2SO_4 in 120 ml water and the resultant precipitate of BaSO_4 was removed by centrifugation. The deep violet aqueous HMnO_4 supernate was transferred to a 500-ml round bottom 24/40 s.t. flask. Up to this point, all solutions and operations were held below 1°C . The flask contents were promptly frozen by immersion in a -75°C CO_2 -acetone bath, then transferred immediately to a vacuum system, the vacuum was applied, and the flask was surrounded by an ice-water bath at 0°C . The vacuum system, operating through successive -75° CO_2 -acetone and -193° liquid N_2

traps, was capable of maintaining a $1\text{-}\mu$ vacuum. However, the steady-state pressure in the flask was determined primarily by the vapor pressure of its contents. Ice immediately appeared on the -75°C trap, indicating the removal of water.

After 10-20 hours a violet color began to appear on the -75° trap ice surface, indicating removal of HMnO_4 . Part of the system was then shunted through a U-tube in a -75° bath and removed for analysis.

After 30-80 hours all the volatile HMnO_4 had been removed and contents of flask and traps were removed for analysis. The nonvolatile residue was extracted to yield 1.2% MnO_2 and 98.8% aqueous HMnO_4 .

Analysis of the solution showed the soluble fraction of the nonvolatile residue to be anhydrous HMnO_4 with an over-all yield of $51 \pm 1\%$.

Analytical difficulties limited precision to a few % for the volatile fraction, but within these limits it analyzed for $\text{HMnO}_4 \cdot 2\text{H}_2\text{O}$ with a yield of $49 \pm 1\%$. Thus, the over-all procedure was close to quantitative.

The anhydrous HMnO_4 proved to be a violent oxidizing agent. Every organic material tried (alcohols, alkanes, benzene hydrocarbons, greases, cycloalkanes, alkyl amines or amides, ethers etc.) burst into flame instantly on contact except CCl_4 , CHCl_3 and CH_2Cl_2 . The latter (CH_2Cl_2) was not as inert as the other two, and reacted slowly. HMnO_4 was very soluble in water, slightly soluble in fluorinated hydrocarbons (perfluorodecalin and CCl_3CF_3) and insoluble in CCl_4 or CHCl_3 . On one occasion, it decomposed vigorously at 20°C but not explosively.† On all other occasions decomposition required 10-30 minutes. It was stable at -75° with less than 1% decomposition for at least 3 days, and was fairly hygroscopic.

† This may have been due to the inadvertent addition of some organic material, e.g., stopcock grease.

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While considerable intriguing chemistry remains to be done with this compound, the present preparation should be adequate for biological trials, since 8–10 g can be stored at -75° and volatilized in small amounts at 0° when needed for fixation of electron microscope preparations.

CONCLUSIONS

A preparation for the hitherto unreported HMnO_4 and $\text{HMnO}_4 \cdot 2\text{H}_2\text{O}$ is given. The latter is sufficiently volatile and stable at 0° to be used as a vapor phase electron microscopy fixative analogous to vapor phase OsO_4 .

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DESIGN AND CONSTRUCTION OF A CENTRIFUGE FOR LARGE FRAGILE CELLS

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When large batches of *Paramecia* (more than 100,000 cells) are centrifuged in conventional centrifuges, extensive lysis occurs, typically lowering the recovery to around 30%. An easily constructed centrifuge with a cylindrical plastic head operated at low

tively, the following three considerations are paramount:

1. High accelerations are unnecessary. Living *Paramecium bursaria* will sediment quantitatively during a few minutes at 5 times gravity. Low ac-

FIG. 138.—Schematic illustration of cylindrical centrifuge assembly. The supernatant core of fluid and rind of cells are represented only in the cross-sectional view. Note that the suction scoop hole is aimed toward the oncoming fluid.

accelerations permits centrifugation of large batches of *Paramecia* with about 90% recovery, even after exhaustive washing. In the construction of a centrifuge able to handle large quantities of fragile cells effec-

celerations are advantageous both for cell survival and the construction of the rotor.

2. The supernatant must be removed without decelerating the rotor since swimming ciliates readily resuspended during deceleration.
3. The rotor must have a *pellet* area large enough

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that the sedimented cells will not form a deep layer.

Construction of the Centrifuge

A hollow cylinder placed on an adapted centrifuge rotor so as to rotate about its (vertical) axis can

FIG. 139.—The plastic cake box rotor, capacity 3 l, diameter 24 cm, height 8 cm.

FIG. 140.—The glass low-form culture flask rotor, capacity 2 l.

FIG. 141.—The intake end of two fluid scoops with turbulence and splashing suppressors.

satisfy the above requirements. If a hole is made in the center of the top, the supernatant can be removed with a stationary suction tube while the cylinder is rotating (Figure 138). We found that a clear plastic cylindrical container, such as a cake box as in Figure 139 (holding about 3 l), could be used very satisfactorily at the low rpm required. Alternatively, a 2500-ml low-form culture vessel from which the neck has been removed can be used as a rotor (Figure 140). Any standard centrifuge can be fitted with a platform which will support the rotor and hold it in a centered position.

Operation of the Centrifuge

The rotor can be filled before or after it is placed in the adapter. Cell suspensions may even be added after the rotor is at operating speed. The supernatant is removed while the rotor is at operating speed with a curved suction tube attached to a trap (Figure 138). A glass nose-cone overriding the crescent-shaped intake scoop, as shown in Figure 141, suppresses splashing, but is not necessary for high recoveries. With *P. bursaria*, supernatant removal is begun about two minutes after the rotor has reached full speed (250

rpm, $3-8 \times g$), by which time the supernatant is essentially cell-free. Operation requires only a few minutes, and the system is easily cleaned. The inexpensive heads can be instantly interchanged, and cultures can even be centrifuged in their original low-

form glass culture vessels. This system, which should be applicable to any large, fragile cells, permits timed experiments that are usually impossible because of the need for exhaustive washing or concentration from large initial volumes.

ELECTRON MICROSCOPIC AUTORADIOGRAPHY OF ALPHA PARTICLES AND PROTONS*

Arthur Lindenbaum and Richard E. Alper

The technique of autoradiography was used in conjunction with electron microscopy to make a high resolution study of the characteristics of tracks produced in silver emulsion by high-energy alpha particles and protons.

While the tracks produced by the different particles

have many characteristics in common, differences were noted, probably based on energy release.

Further work of this nature may allow more precise localization of intra- and extracellularly deposited isotopes in animal tissues and a better correlation between irradiation and pathologic effects. High-magnification techniques may also have some application in the photographic interpretation of subatomic particles.

* Abstract of paper to be published in *Atompraxis*.

Enzymology

THE METABOLIC RELATIONSHIP OF METHIONINE TO ITS SULFONIUM DERIVATIVES

Stanley K. Shapiro, Aldona Almenas, and John F. Thomson

PURPOSE AND METHODS

Methionine is one of the so-called essential amino acids; it also serves as an indirect source of methyl groups for many biological compounds. It has been established that adenosylmethionine is the most important metabolic source of intact methyl groups in the biosynthesis of numerous biological compounds, including phospholipids, sterols, and methylated nucleic acids.⁽¹⁾ This project is concerned with the metabolism of adenosylmethionine and its demethylated product, adenosylhomocysteine, with particular emphasis on their relationship to the mechanism of methionine biosynthesis.

The participation of adenosylmethionine in the biosynthesis of methionine is now generally recognized. However, its function as a direct methyl donor has been established only in microbial cells, seeds of higher plants, and rat liver.⁽¹⁾ The participation of adenosylmethionine in methionine biosynthesis was first demonstrated in this laboratory several years ago. At that time we described the enzyme, adenosylmethionine:homocysteine methyltransferase, which catalyzes the following reaction in *Saccharomyces cerevisiae*:

This is the only known mechanism of methionine biosynthesis from homocysteine in yeast cells (*S. cerevisiae*). The methods of purification and the properties of the adenosylmethionine:homocysteine methyltransferase of yeast cells have been described previously.^(1, 2) The current investigation has provided insight into the kinetics and mechanism of this reaction.

PROGRESS REPORT

It has been demonstrated that the addition of 2-mercaptoethanol to standard reaction mixtures made it possible to study the kinetics of the reaction.⁽³⁾ Pro-

duct-inhibition studies were undertaken to obtain information concerning the order of addition of the two substrates. The reaction velocity was measured in a series of reaction mixtures containing varying amounts of adenosylmethionine (AM) and homocysteine (H), both without product and with either methionine or adenosylhomocysteine (AH) added. In the presence of methionine, a plot of $1/v$ against $1/AM$ showed a marked departure from linearity (Figure 142, Curves 1 and 2), whereas a plot of $1/v$ against $1/H$ showed a change in slope but no change in intercept in the presence of methionine, so that when these intercepts were then plotted against $1/AM$ (Figure 143, Curve 3), the line obtained was virtually identical with that obtained in the absence of methionine. In the presence of the other product, AH, the intercepts of both $1/v$ vs. $1/H$ and $1/v$ vs. $1/AM$ plots were altered; the secondary plots of these intercepts against the other substrate showed that there is a straight line relationship for the intercept plot against $1/AM$ (Figure 143, Curve 1) but not against $1/H$ (Figure 144, Curve 1).

These data are consistent with several mechanisms involving an ordered binding, with homocysteine the first substrate bound and adenosylhomocysteine the first product released. There is also evidence of product inhibition or both product and substrate inhibition, but the data are not precise enough to distinguish among the various possibilities.

It may be noted in Figure 142 that Curves 3 and 4 are nearly parallel. This observation implies that the coefficient of the reciprocal of the product of the substrates, ϕ_{12} in the Dalziel equation⁽⁴⁾

$$\frac{E_0}{v} = \phi_0 + \frac{\phi_1}{[H]} + \frac{\phi_2}{[AM]} + \frac{\phi_{12}}{[H][AM]}$$

is essentially zero.

On the basis of Curve 2 in Figure 143 and Curve 2 in Figure 144, the Michaelis constants can be estimated

FIG. 142.—Double reciprocal plots of initial reaction velocities against concentrations of adenosylmethionine. AM, adenosylmethionine, μ moles per ml; v , reaction velocity, μ moles of methionine per 0.5 ml in 30 min. Solid symbols (Curves 1 and 2), 0.5 μ mole of methionine per ml; open symbols (Curves 3 and 4), no methionine. Triangles (Curves 1 and 3), 1.0 μ mole of homocysteine per ml; circles (Curves 2 and 4), 4.0 μ moles of homocysteine per ml. Pairs of curves with three other concentrations of homocysteine were also obtained but were not plotted for the sake of clarity. The lines that did not deviate significantly from linearity were fitted by the method of least squares.

FIG. 143.—Intercepts of $1/v$ vs. $1/H$ plots as a function of $1/AM$. AM, adenosylmethionine, μ moles per ml; H, homocysteine, μ moles per ml; v , reaction velocity, μ moles of methionine per 0.5 ml in 30 min. Curve 1, 0.47 μ mole of adenosylhomocysteine per ml; Curve 2, no adenosylhomocysteine. Curve 3 (data from another experiment): circles, no methionine; triangles, 0.5 μ mole of methionine per ml. The lines were fitted by the method of least squares.

as $3.2 \times 10^{-4} M$ for homocysteine and $8.6 \times 10^{-4} M$ for adenosylmethionine.

The separation cell method of Yphantis and Waugh^(5, 6) was used to determine the sedimentation

coefficient ($s_{20,w}$) of adenosylmethionine:homocysteine methyltransferase. From the value $3.3 \pm 0.6 \times 10^{-5}$ sec it was estimated that the molecular weight of the enzyme is of the order of 30,000. This agrees with a

FIG. 144.—Intercepts of $1/v$ vs. $1/AM$ plots as a function of $1/H$. Abbreviations are the same as in Figure 143. Curve 1, 0.47 μ mole of adenosylhomocysteine per ml; Curve 2, no adenosylhomocysteine. Curve 2 was fitted by the method of least squares.

similar estimate based on gel filtration of the enzyme on a Sephadex G-75 column.⁽²⁾

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THE BIOSYNTHESIS OF METHIONINE IN ESCHERICHIA COLI

Edward Balish, Stanley K. Shapiro, and Dimis J. Ehninger

PURPOSE AND METHODS

This report is a continuation of the project concerned with the metabolism of adenosylmethionine and adenosylhomocysteine and their relation to methionine biosynthesis.⁽¹⁾ The current investigation is directed toward the role of these compounds in the biosynthesis of methionine in *Escherichia coli* and other microorganisms with particular emphasis on the control mechanisms involved. The isotopic methods employed to quantitate methionine production by cell-free extracts have been described in detail previously.⁽²⁾

GRESS REPORT

Methionine biosynthesis has been studied with cell-free extracts prepared from *E. coli* Texas and *E. coli*

Texas M, a methionine auxotroph. The latter organism is unusual in that it represents one of the few auxotrophic mutants that can satisfy its methionine require-

TABLE 78. EFFECT OF INCUBATION MEDIUM ON TRANSMETHYLASE ACTIVITY*

Source of enzyme	Methyl donor	Peptonized milk	Minimal medium + methionine
<i>E. coli</i> (Texas)	Methylmethionine	215	93
<i>E. coli</i> (Texas M)	Methylmethionine	305	109
<i>E. coli</i> (Texas)	Adenosylmethionine	45	22
<i>E. coli</i> (Texas M)	Adenosylmethionine	65	22

* μ moles methionine/mg protein/hour.

ment with the methyl donors, adenosylmethionine or methylmethionine.

Table 78 shows that cell-free extracts of *E. coli* Texas and *E. coli* Texas M form methionine by transferring a methyl group from adenosylmethionine or methylmethionine to homocysteine. *E. coli* Texas M forms more methionine via this transmethylation than does the wild type (Texas). The amount of methionine formed by cell-free extracts of both organisms was greater when the organisms were grown in 1.5% Difco peptonized milk than when the cells were grown in a glucose-salts-methionine medium (Table 78).

This observation led to the hypothesis that the homocysteine methyltransferase may be inducible. To explore this possibility, cells which had originally been grown in peptonized milk were suspended in a glucose-salts medium with methionine, and cell-free extracts were prepared from cells collected at various time intervals. The remaining cells were then harvested, washed and divided into two parts. One-half of the cells was suspended in a glucose-salts-homocysteine medium and the second half in a glucose-salts-homocysteine medium containing chloramphenicol.

The results of a typical experiment are shown in Figure 145. It can be seen that methyltransferase activity decreased in the presence of methionine. The cell-free extracts prepared from cells suspended in a glucose-salts-homocysteine medium showed a significant increase in their capacity to form methionine via transmethylation with methylmethionine as the methyl donor and homocysteine as the methyl acceptor. The presence of chloramphenicol appeared to prevent a

similar increase in activity although there was a slight increase in activity which was greater than that observed with extracts of cells suspended in the presence of methionine. It appears that protein synthesis is probably involved in the increased enzymatic activity shown by cells in the glucose-salts-homocysteine medium.

The following reactions represent the current concept of the over-all pathway for the biosynthesis of methionine in microorganisms:

The immediate precursor of homocysteine is cystathionine. Cell-free extracts prepared from *E. coli* Texas M form methionine from cystathionine as shown in Table 79. The cells used for these studies were grown in peptonized milk. However, methionine formation with cystathionine as a methyl acceptor also increases with cell-free extracts of cells grown in peptonized milk and then suspended in a glucose-salts-cystathionine medium (Table 79). The latter reaction has not been demonstrated with extracts of cells grown in peptonized milk and suspended in a glucose-salts medium with either homocysteine or methionine added. This seems to indicate that methionine and homocysteine may repress cystathionase activity which is required to form methionine from cystathionine.

FIG. 145.—Methyltransferase activity of cell-free extracts prepared from *E. coli* Texas M grown in peptonized milk and suspended in minimal medium with homocysteine or methionine added.

TABLE 79. EFFECT OF CYSTATHIONINE ON TRANSMETHYLASE ACTIVITY*

Source of enzyme	Methyl acceptor	Original activity	After treatment†
<i>E. coli</i> (Texas M)	L-Homocysteine	305	490
<i>E. coli</i> (Texas M)	DL-Cystathionine	55	300

* μ moles methionine/mg protein/hour.† One hour exposure to minimal medium + DL-cystathionine (1.0 μ mole/ml).

CONCLUSIONS

Homocysteine and cystathionine do not support the growth of *E. coli* Texas M (Table 80). Nevertheless, when this organism is suspended in a glucose-salts-homocysteine or glucose-salts-cystathionine medium, there is an increase in methionine formation via transmethylation. This supports the hypothesis that the homocysteine methyltransferase of *E. coli* Texas M is inducible. It also suggests that the genetic block in this organism does not involve the enzyme. Since this mutant grows on the methyl donors, adenosylmethionine and methylmethionine, as well as methionine

TABLE 80. GROWTH RESPONSES OF *E. coli* TEXAS M

Supplement (400 μ moles/ml)	O.D. ₅₄₀
None	0
Adenosylmethionine*	0.39
Methylmethionine	0.59
L-Methionine	0.64
D-Methionine	0.65
L- or D-Homocysteine	0.01
L- or D-Homocysteine thiolactone	0.01
DL-Cystathionine	0.01

* Concentration, 166 μ moles/ml.

(Table 80), it is possible that it is actually a methyl donor mutant rather than a methionine mutant.

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THE BIOSYNTHESIS OF METHIONINE IN JACK BEAN MEAL

Lila Abrahamson and Stanley K. Shapiro

PURPOSE AND METHODS

Homocysteine methyltransferases which catalyze the transfer of a methyl group from methylmethionine or adenosylmethionine to homocysteine have been described in cell-free extracts of microorganisms,⁽¹⁾ rat liver,⁽²⁾ and plants.⁽³⁾ It has also been reported that methylmethionine occurs in relatively large quantities in cabbage⁽⁴⁾ and growing jack bean plants.⁽⁵⁾ These are the first examples demonstrating homocysteine methyltransferase activity in the same organisms where the methyl donor occurs in detectable quantities during some phase of the life cycle. It, therefore, seemed worth while to study the homocysteine methyltransferase of jack bean meal in a partially purified state. The methods of enzyme assay and enzyme purification were adapted from those described by Shapiro *et al.*⁽¹⁾

PROGRESS REPORT

Column chromatography

A 35-45% ammonium sulfate precipitate regularly yielded a product which had a specific activity 10-15 times that of the initial extract. One ml of this material

containing 71 mg protein was placed on a column of DEAE-Sephadex G-50, and enzyme activity was eluted with the 0.02 M phosphate-0.5 M NaCl buffer. This eluate had a specific activity 109 times that of the starting material. No further purification was attempted since the quantity of total protein recovered was small (Table 81).

As an alternate procedure for DEAE-Sephadex chromatography, 1.5 ml of the ammonium sulfate precipitate was placed on a Sephadex G-75 column.

TABLE 81. PURIFICATION OF HOMOCYSTEINE METHYLTRANSFERASE OF JACK BEAN MEAL

Procedure	Specific activity*	Purification factor	% yield
Buffer extraction	19		100
Ammonium sulfate fractionation	272	14	30
DEAE-Sephadex G-50 chromatography†	2079	109	14
Sephadex G-75 chromatography†	4711	248	9

* μ moles methionine/mg protein/hour incubation.

† The starting material for this procedure was the 35-45% ammonium sulfate fraction.

FIG. 146.—Chromatography of homocysteine methyltransferase (35–45% ammonium sulfate precipitate) on Sephadex G-75. The solid line represents the specific activity of the enzyme as $\mu\text{moles methionine/mg protein}$; the protein recovered is shown as a dashed line. A total of 81 mg of protein in 1.5 ml was added to the column ($0.9 \times 200 \text{ cm}$), and 3-ml fractions were collected at the rate of 1 ml/6 minutes.

Enzyme was eluted after the major protein peak (Figure 146), and this suggests that the enzyme protein has a molecular weight under 40,000. In the course of the elution, the enzyme was purified 17-fold compared with the material placed on the column or 248-fold-compared with the original extract (Table 81).

Kinetics

Lineweaver and Burk double reciprocal plots were employed for calculation of the Michaelis constants. The K_m for methylmethionine was $5.5 \times 10^{-5} M$, and the K_m for homocysteine was $4.1 \times 10^{-5} M$.

Studies on the effect of temperature revealed that optimum enzyme activity occurred at 60°C . However, standard reaction mixtures were incubated at 37°C because the substrates were not stable at the higher temperatures. The reaction rate between 30° and 50°C was plotted, and from the slope of the line, the activation energy was calculated to be 16,200 calories. Similarly, the standard assay mixture was adjusted to pH 6.9 although the optimum activity of the enzyme appeared to be at pH 7.9. At the latter pH, methylmethionine was not stable and contributed high control

values when enzyme was not added to the reaction mixture.

Stability and inhibition

The time course of the standard methyltransferase reaction was studied at three concentrations of an enzyme preparation containing 2.4 mg protein per milliliter with a specific activity of 376 $\mu\text{moles methionine per milligram protein per hour}$. A linear relationship was shown at all three concentrations of enzyme, indicating that the enzyme was stable for 2 hours at 37°C and that there was no apparent product inhibition at the levels of methionine produced during the extended incubation, i.e., up to 333 $\mu\text{moles per 0.48 mg protein per 2 hours}$.

Product inhibition was tested further by adding methionine to the standard assay mixture containing 0.1 ml of the above enzyme preparation; the results obtained with the enzyme of jack bean meal are shown in the top line of Figure 147. No inhibition occurred until more than 500 μmoles of methionine were present. Beyond this level of methionine, inhibition of enzyme activity was still quite low as compared with the inhibition obtained with a similar enzyme from *Saccharomyces cerevisiae* as shown by the lower line of Figure 147.

The chelating agent, EDTA, has been shown to inhibit the homocysteine methyltransferases of various microorganisms but not that of rat liver.⁽⁶⁾ The par-

FIG. 147.—Inhibition of homocysteine methyltransferase activity by the product, methionine. Standard assay mixture contained various concentrations of methionine. The solid line represents the activity of the enzyme of jack bean meal; the dashed line represents the activity of the enzyme of *S. cerevisiae*.⁽⁷⁾

TABLE 82. EFFECT OF EDTA ON HOMOCYSTEINE METHYLTRANSFERASE ACTIVITY

Source of enzyme	EDTA concentration, <i>M</i>	Methionine produced, μ moles/0.5 ml	Enzyme activity,* %
Jack bean meal extract, partially purified	0	0.6	100
	5×10^{-4}	0.6	100
	5×10^{-2}	0.5	83
Pea meal extract, not purified†	0	3.2	100
	8×10^{-3}	0.4	13

* Percentage of enzyme activity in the absence of EDTA.

† From Turner and Shapiro.⁽³⁾

tially purified homocysteine methyltransferase of jack bean meal was not inhibited by EDTA at a level of 5×10^{-4} *M*, and only 17% inhibition was caused by EDTA at a level of 5×10^{-2} *M* (Table 82). In contrast, EDTA at a level of 8×10^{-3} *M* resulted in 87% inhibition of homocysteine methyltransferase activity of an extract of pea seeds.⁽³⁾

The addition of a reducing agent, 2-mercaptoethanol, to the reaction mixture did not increase the rate of activity of the enzyme of jack bean meal, even at low levels of homocysteine. At similar low levels of homocysteine, 2-mercaptoethanol produced a marked increase in activity of the homocysteine methyltransferase of *S. cerevisiae*.⁽⁷⁾

Methyl donor specificity

Two methyl donors which substituted for methylmethionine with the homocysteine methyltransferase of jack bean meal were adenosylmethionine and dimethyl- β -propiothetin. Dimethylacetothetin, however, was not active in this system. Of the active methyl donors, adenosylmethionine showed approximately 10% of the activity of methylmethionine, whereas dimethyl- β -propiothetin and methylmethionine showed the same activity (Table 83).

CONCLUSION

The homocysteine methyltransferase of jack bean meal, with its many distinctive properties, catalyzes the biosynthesis of methionine in a higher plant system.⁽⁸⁾ In recent years a great deal of attention has been focused on methionine biosynthesis in microorganisms and mammalian liver.⁽⁹⁾ However, the principal

TABLE 83. COMPARISON OF VARIOUS METHYL DONORS FOR THE HOMOCYSTEINE METHYLTRANSFERASE OF JACK BEAN MEAL

Methyl donor	Specific activity*
S-Methyl-L-methionine	170†
S-Adenosyl-L-methionine	16
Dimethylacetothetin	0
Dimethyl- β -propiothetin	181

* μ moles methionine/mg protein/hour incubation.

† This value represents half the total methionine yield because only one-half of the recovered methionine results from the transfer of a methyl group from S-methyl-L-methionine to L-homocysteine.

nutritional source of methionine in mammalian metabolism is derived from plant proteins, and it, therefore, seems very important to investigate the mechanism of methionine biosynthesis in plants.

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EFFECT OF COMPOUNDS RELATED TO AMINOTRIAZOLE ON BLOOD AND LIVER CATALASE. II.

Robert N. Feinstein and Judith B. Howard

PURPOSE AND METHODS

In the 1964 Annual Report of this Division,⁽¹⁾ we described a continuing search for effective catalase inhibitors, in the hope of obtaining a compound capable of inhibiting erythrocyte catalase *in vivo*. Although several compounds are known effective inhibitors of the catalase of solid tissues, none is yet known capable of inhibiting catalase in the intact erythrocyte.

PROGRESS REPORT

Since the last report, the following compounds have been tested:

Purchased from Aldrich Chemical Company:

3,4,5,6-tetrahydro-2-pyrimidine thiol

trithiocyanuric acid

N-acetamidorhodanine

2-amino-4,6-pyrimidine dithiol

aminopyrazine

3-amino-1,2,4-triazine

N-2-thiazolyl acetoacetamide

2-amino-2-thiazoline

Purchased from Gallard-Schlesinger Chemical Manufacturing Corporation:

2-amino-5-chlorothiazole

2-amino-5-mercaptol-1,3,4-thiadiazole

aminourazole

Purchased from FMC Corporation:

cyanuric acid

These compounds were administered intraperitoneally at the highest practicable (i.e., nonlethal) dose, and blood and liver catalase activities were assayed after one hour. Of the compounds listed, none affected either liver or blood catalase.

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THE SYNTHESIS OF PROPIONATE BY THE PROPIONIBACTERIA

Edward J. Wawszkiewicz and Robert W. Swick

PURPOSE AND METHODS

Propionate plays an important role in a number of biological processes. It is, for example, produced by bacteria in the digestive tract of ruminants, which can oxidize it as an energy source or incorporate it into higher, odd-numbered fatty acids. Conversely, propionate is formed by the beta oxidation of higher, odd-numbered fatty acids. It can also be formed by the degradation of branched chain aliphatic amino acids and by the reduction of certain intermediates of carbohydrate metabolism. The pathway of propionate formation by the propionibacteria has been extensively investigated: the penultimate step is the transfer of a carboxyl group from methylmalonyl-coenzyme A to pyruvate with the concomitant formation of oxalacetate and of propionyl-coenzyme A. Methylmalonyl-coenzyme A arises from succinyl-coenzyme A by an isomerization reaction, the detailed mechanism of which remains obscure. Free propionate is not involved in the reaction, nor is acrylate, another substance once thought likely to take part. However, we found that acrylate can be esterified to acrylyl-coenzyme A and reduced to propionyl-coenzyme A

with NADH_2 .⁽¹⁾ This sequence had not been demonstrated in these organisms previously and is of current interest because it implies a pathway of propionate synthesis in the propionibacteria alternate to that involving methylmalonyl-coenzyme A.

A spectrophotometric assay of propionyl-coenzyme A dehydrogenase, the enzyme which catalyzes the reduction of acrylyl-coenzyme A to propionyl-coenzyme A, has been developed to follow the enrichment of the enzyme. We are attempting to purify this enzyme and to ascertain the role of acrylyl-coenzyme A in the metabolism of these microorganisms.

PROGRESS REPORT

We have subjected cell-free extracts of *Propionibacterium shermanii* to various protein-fractionation techniques (Table 84).

Our attempts to increase the resolving power of zone electrophoresis by using blocks of polyacrylamide-beads of various porosities have been unsuccessful. However, we are led to believe that a high degree of enrichment, perhaps ultimate purification of the enzyme, can be achieved by subjecting our best

TABLE 84. PURIFICATION OF PROPIONYL-COENZYME A DEHYDROGENASE

Fraction	Specific activity, μ moles NADH ₂ oxid./mg protein/ 10 min
Cell-free extract	0.431
Ammonium sulfate, 50-75%	0.988
DEAE-cellulose column	4.01
Ammonium sulfate, 58-75%	8.66
Starch block electrophoresis, pH 8.6	15.44

The material obtained after the second precipitation with ammonium sulfate separated into 16 components in analytical polyacrylamide-gel electrophoresis columns, and that recovered from starch block electrophoresis still contained 6 components.

ERYTHRITOL METABOLISM BY PROPIONIBACTERIUM PENTOSACEUM

Edward J. Wawszkiewicz

PURPOSE AND METHODS

Propionibacterium pentosaceum is the only microorganism known capable of anaerobic growth using the four-carbon polyalcohol, erythritol, as sole source of carbon. The major products of its fermentation of erythritol are propionic, succinic, acetic and formic acids. Suspensions of "sloppy-dried" cells, that is to say of cells dried in thin layers in vacuum over phosphorus pentoxide at 0°, metabolize erythritol, in the presence of sodium pyruvate and sodium fluoride, with a concomitant uptake of inorganic phosphate from the medium and the accumulation of difficultly-hydrolyzable phosphate esters. When randomly labeled erythritol-C¹⁴ is thus metabolized, labeled glycerol-1-phosphate, glyceric acid-3-phosphate, erythritol-1-phosphate, pentose phosphates, glucose-6-phosphate, fructose-6-phosphate, mannose-6-phosphate, fructose-1,6-diphosphate and sedoheptulose-7-phosphate accumulate. Erythritol-1-phosphate accumulates more rapidly than do the other compounds and, even under conditions in which its dephosphorylation is inhibited, it is converted to the same products as is erythritol. Also, in short term experiments, the addition of unlabeled L-erythritol-1-phosphate to cell preparations actively metabolizing erythritol-C¹⁴ reduces the amount of label normally found as glyceric acid-3-phosphate. This evidence supports the view that in *Propionibacterium pentosaceum* erythritol metabolism proceeds via a series of phosphorylated intermediates, of which L-erythritol-1-phosphate is the primary member. Indirect evidence has suggested that L-erythrulose-1-phosphate is the next intermediate in the reaction sequence. The isolation

preparations to analytical polyacrylamide-gel electrophoresis on a preparative scale. We are currently constructing an apparatus for this purpose.

Only 2, *Propionibacterium freudenreichii* and *Propionibacterium shermanii*, of the 15 species of propionibacteria tested possess propionyl-coenzyme A dehydrogenase. Our latest work indicates that, contrary to the conclusions of a preliminary experiment, in *Propionibacterium shermanii*, at least, the enzyme is constitutive.

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tion of labeled formaldehyde from the reaction mixtures has suggested further that the L-erythrulose-1-phosphate formed undergoes cleavage at the 3 and 4 positions.

From Professor Clinton Ballou* we have recently obtained some synthetic L-erythrulose-1-phosphate and have prepared some L-erythrulose-1-phosphate-C¹⁴, specifically labeled in the 4 position, by reacting labeled formaldehyde with unlabeled dihydroxyacetone phosphate in the presence of an enzyme from rat liver. With these two materials at hand we have studied directly the role of L-erythrulose-1-phosphate in erythritol metabolism by *Propionibacterium pentosaceum*.

PROGRESS REPORT

Using cell-free extracts we have now shown that: a) At pH 9 L-erythritol-1-phosphate can act as substrate for NADH formation, while erythritol itself cannot. b) Conversely, at pH 7, the addition of L-erythrulose-1-phosphate to cell extracts containing NADH results in the rapid oxidation of the NADH with a simultaneous production of erythritol phosphate. These results suggest the existence of a new enzyme, L-erythritol-1-phosphate dehydrogenase. The only other enzyme known to be capable of catalyzing the reduction of L-erythrulose-1-phosphate, glycerol phosphate dehydrogenase from liver, catalyzes its reduction to threitol phosphate. c) *Propionibacterium pentosaceum* is capable of producing L-erythrulose-1-phosphate from formaldehyde and dihydroxyacetone phosphate, this

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being a consequence of the reversibility of the postulated splitting reaction.

We intend to isolate labeled L-erythrulose-1-phos-

phate directly from *Propionibacterium pentosaceum* cell suspensions metabolizing erythritol-C¹⁴ and to purify L-erythritol-1-phosphate dehydrogenase.

THE METABOLISM OF MITOCHONDRIAL PROTEINS

Robert W. Swick

PURPOSE AND METHODS

We are interested in measuring, in an unequivocal fashion, the metabolic turnover of various protein constituents of the liver; our attention has been focused on the mitochondrial proteins. Elucidation of the pattern of replacement of the individual enzymes and structural membranes may lead to a better understanding of the normal dynamics of this particle. The first experiments, using isotopes, showed similar turnover rates for the purified structural protein and several crude fractions of the remaining proteins. This suggested that the mitochondrion may be replaced as a unit.

Ideally, one would like to measure the turnover rates of a number of pure proteins. Few mitochondrial enzymes have been purified to homogeneity, and the amount of material necessary for such isotope experiments is substantial. A simpler approach, one which would give an independent measure of the turnover rates as well, would be to measure the rate of change of enzyme activity in mitochondria during metabolic manipulation of the host. If the above hypothesis of mitochondrial formation is valid, the rate of change of enzyme activity should be similar to the rate of synthesis of new mitochondria as measured by the incorporation of the isotope.

Estimates of the renewal rates of mitochondrial proteins were made by the continuous exposure of rats to C¹⁴O₂ through the addition of CaC¹⁴O₃ to the diet which was then fed fractionally 24 times a day. The turnover rates of 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 using the following equation:

$$R = U(1 - e^{-kt}),$$

where R is the specific activity of the fraction, U is the specific activity of urea at equilibrium, and k is the turnover rate in days⁻¹.

This expression gives a valid estimate of the turnover rate when the quantity of the precursor is relatively very small, is rapidly renewed, and achieves its

ultimate specific activity in a time which is short, relative to the turnover rates being measured, and which is largely unaffected by the reutilization of unlabeled compounds.⁽¹⁾ Such an expression should also be applicable to the estimate of the renewal rates of mitochondria from the rate of change of enzyme levels. The (polysome) precursor pool is small, is turning over very rapidly, and probably reaches its ultimate specific activity very quickly, relative to the rate of mitochondrial renewal. Although no direct evidence is available, it appears unlikely that there is a significant reincorporation of liberated mitochondrial enzymes into newly formed mitochondria. Estimates can be made either from the rise in activity during induction

$$(A_t - A_0) = (A_e - A_0)(1 - e^{-kt})$$

or from the fall in activity after removal of the stimulus

$$(A_t - A_0) = (A_e - A_0)e^{-kt},$$

where A_0 is the initial or normal specific enzyme activity, A_e is the specific activity at equilibrium after stimulus, and A_t is the specific activity at any time, t , between.

Young female rats of the SD/ANLspf strain were placed initially on diets containing either 12% or 90% casein. After 10-14 days, the specific activities of mitochondrial alanine aminotransferase (AAT), ornithine aminotransferase (OT), and ornithine transcarbamylase (OTC) were constant. Animals were then changed to the other diet and sacrificed at intervals for two weeks. Enzyme activities were determined spectrophotometrically⁽²⁾ or colorimetrically.^(3, 4)

PROGRESS REPORT

Kinetics of Mitochondrial Renewal

The activity of the three enzymes increased in the rats fed the high protein diet. The extent of the increase was related inversely to the initial specific activities (Table 85).

Also in Table 85 are the average rates of change of activity of the three enzymes. Since there was consid-

erable variation in the estimates obtained at different times, the differences between enzymes are not significant. It appears that the rates of decrease in activity were more rapid than the rates of increase. This will require further investigation. All values differ rather markedly from estimates of the turnover rate of mitochondrial proteins measured by isotope incorporation: 0.20. The latter measurements were made with animals in dynamic equilibrium. However, estimates made in animals fed diets high in protein for long periods were similar. For direct comparison, isotope incorporation will have to be measured in animals undergoing changes in enzyme activity levels. If these differences in renewal rates persist, the current idea regarding the formation of mitochondria and the stability of the enzyme complement during the lifespan will have to be revised.

Berlin and Schimke⁽⁵⁾ have shown that the absolute amount of enzyme protein synthesized in response to a stimulus is the same for each of four enzymes whose initial activities and turnover rates differ widely. The apparent specificity of response of some enzymes is not due to a specific stimulation of their synthesis but is a reflection of their rapid turnover rates. In the present experiments, we observed varying degrees of stimulation of enzymes with similar turnover rates. Since their renewal rate may be fixed by that of the mitochondrion, this does not preclude a similar increase in the absolute amount of each protein synthesized. Further investigation is obviously required.

Mitochondrial Alanine Aminotransferase

A number of investigators have failed to find an effect on the activity of mitochondrial AAT of feeding diets high in protein or of the injection of corticosteroids. Only in our laboratory has an increase (two- to fourfold) in the activity of this enzyme been observed. As this is the first instance of a mitochondrial enzyme responding to corticosteroid treatment, further investigation is imperative. The following evidence, in a continuing program, has recently been garnered.

The rate of increase in activity of AAT in the mitochondria during stimulation was like that of other mitochondrial enzymes (Table 85). The rate of change of the soluble isozyme, also measured in these experiments, was slower: $k_{(up)} = 0.20$; $k_{(down)} = 0.32$. In addition, mitochondria from normal and from treated rats have been isolated by differential centrifugation, using pure sucrose solutions and sucrose solutions enriched in the soluble isozyme prepared from normal rats fed from rats fed high protein diets or injected with prednisolone. There was no significant difference in any of the specific activities of the mitochondrial

TABLE 85. KINETICS OF MITOCHONDRIAL RENEWAL AS MEASURED BY CHANGES IN THE LEVEL OF THREE ENZYMES

Enzyme	A_0 , μ moles/min/mg	Increase, %	k_{up} , days ⁻¹	k_{down} , days ⁻¹
OT	0.0095	1300	0.36	0.50
AAT	0.025	370	0.32	0.46
OTC	1.8	202	0.24	0.44

TABLE 86. DISTRIBUTION OF AAT AND GDH

Treatment	AAT specific activity	Median fraction of activity	
		AAT	GDH
Control	0.024	10.68 $\pm$ 0.13	11.00 $\pm$ 0.09
High casein	0.049	17.56 $\pm$ 0.19	17.58 $\pm$ 0.10
Prednisolone	0.044	16.44 $\pm$ 0.13	17.30 $\pm$ 0.12

preparations from any given animal, control or experimental. These two experiments suggest that it is unlikely that the observed increase in activity in the mitochondria of treated rats is the result of contamination or incorporation of the soluble isozyme during preparation of the particulate fraction.

Gradient centrifugation of mitochondrial fractions were performed using a zonal centrifuge, model A-12. Glutamate dehydrogenase (GDH) was used as a marker enzyme, since it is known to be associated only with the mitochondria. The activities of the two enzymes in the fractions recovered were compared by calculating the cumulative normal curves. The results are shown in Table 86.

Under conditions where the specific activity of AAT was twice that of untreated rats, there was neither a separation of AAT activity from that of GDH nor the appearance of a second peak of AAT activity. Therefore, it seems unlikely that some other particle rich in AAT activity, e.g., polyribosomes, was included. Contamination of our preparations with microsomes and microbodies was demonstrated, but no AAT activity was associated with them. The difference between the median obtained with control animals and those of the treated animals was significant. Whether this was due to differences in centrifugation or differences between normal and treated animals is currently under investigation.

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STEROL METABOLISM

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PURPOSE AND METHODS

One of the most interesting aspects of the terminal stages of the conversion of lanosterol to cholesterol is the reaction sequence by which the three methyl groups of lanosterol are removed. This work has been mainly concerned with the conditions governing the removal of the 14α -methyl group, since it has been claimed that this is invariably the first step in the sequence. Recently, however, the discovery of the naturally-occurring sterol macedougallin (14α -methyl- Δ^8 -cholestene- $3\beta,6\alpha$ -diol) cast doubt on this proposition, at least as far as plant metabolism is concerned. A series of experiments was, therefore, planned to determine the part played in animal sterol biosynthesis by 14α -methyl sterols, and to this end certain synthetic 14α -methyl sterols were prepared, labeled with tritium, and incubated with homogenates of rat liver.

PROGRESS

A technique for labeling sterols with tritium was worked out and is described in detail elsewhere in this report. It involves oxidation to the corresponding ketone, which is labeled by exchange of its enolic hydrogens for tritium on a column of alumina treated with tritiated water, followed by reduction back to the alcohol with lithium aluminum hydride and purification by digitonin precipitation. Incubations of 14α -methyl- Δ^7 -, and 14α -methyl- Δ^8 -cholestene- 3β -ol which had been labeled in this manner were then carried out, and the presence of labeled cholesterol in the incubation products was demonstrated by two different methods:

- 1) Carrier precipitation of the labeled cholesterol

dibromide followed by crystallization to constant specific activity, and debromination to cholesterol of the same specific activity.

- 2) Hydrogenation of the acetylated incubation product followed by oxidation to remove unreacted starting material, after which the presence of cholestanol could be demonstrated by cochromatography with synthetic cholestanol-1- C^{14} -acetate.

In a two-hour incubation, it was found that 7% of the 14α -methyl- Δ^7 -cholestenol and 15% of the Δ^8 isomer underwent conversion to cholesterol, compared with 25% conversion of Δ^7 -cholestenol, a well-established cholesterol precursor. A suspected intermediate compound, $\Delta^{8(14)}$ -cholestenol, was also incubated under similar conditions and found to undergo no apparent conversion.

CONCLUSIONS

These results show that loss of the 14α -methyl group of lanosterol is not an obligatory first step, but can occur after removal of the 4,4-dimethyl grouping. Since the Δ^8 -isomer is converted in somewhat better yield than the Δ^7 , it is probable that the first step in metabolism of the Δ^7 , is in fact isomerization to Δ^8 . The precise mechanism by which the methyl group is removed is not yet clear, since if it proceeded by the accepted route involving oxidation of the methyl to $-\text{COOH}$ followed by decarboxylation, $\Delta^{8(14)}$ -cholestenol would be expected to be an intermediate, and this is apparently not the case. A synthetic program, designed to produce model compounds with a 14α -carboxyl group, has been initiated, and it is hoped that incubation of these will shed further light on the subject.

Cellular Biochemistry

ACTION OF RIBONUCLEASE PREPARATIONS ON YEAST CELLS AND SPHEROPLASTS

Fritz Schlenk

PURPOSE AND METHODS

In cytological studies on yeast cells and spheroplasts, it was found that viable cells of *Candida utilis* and *Saccharomyces cerevisiae* lose the major part of their ultraviolet-absorbing constituents to the surrounding medium when exposed to ribonuclease. This result is in disagreement with numerous statements in the literature, according to which the yeast cell is impervious to the action of ribonuclease.

The present report deals with the conditions and the enzyme preparations necessary for action on whole yeast cells and spheroplasts. It explains the discrepancies from observations of earlier investigators and describes a simple method of preparing ghosts of yeast cells with low content of ribonucleic acid. The technique is of importance for the selective extraction of cellular components. In addition, several characteristics of the yeast cell surface and of the enzyme ribonuclease have come to light.

PROGRESS REPORT

Washed cells in water suspension (2 mg/ml) are exposed to ribonuclease preparations (0.05 to 0.1 mg/ml). Ultraviolet-absorbing cellular constituents are released from the cells into the medium. Spectrophotometry at 260 $m\mu$ reveals that as much as 80% of the total extractable absorbing material may be found in the suspending water after 30 minutes as judged by comparison with exhaustive extraction by perchloric acid.

Several experimental conditions have to be observed to assure a maximal effect. Buffer solution has to be omitted because cations are highly inhibitory. About 50% reduction of the effect of ribonuclease is observed in presence of 10mM K^+ , 5mM NH_4^+ , 0.25mM Mg^{++} , or 0.05mM Ca^{++} . Most efficient, however, is spermine which inhibits in a concentration of 10^{-3} mM.

Among anions, phosphate at a level of 2 mM blocks ribonuclease above pH 6 completely, while acetate in this concentration is tolerated.

Not less important is the type of ribonuclease and the method by which it has been obtained. Recrystallized pancreatic ribonuclease, isolated by the processes of Kunitz⁽¹⁾ and McDonald,⁽²⁾ is very effective. Chromatographically purified pancreatic ribonucleases A and B were found inactive. However, heating of the

water solutions of ribonuclease A or B for 5 minutes at 70° restored full activity of action on yeast cells. It appears, therefore, that a difference in the conformational structure is decisive for the effect of the enzyme.

After removal of the yeast cell wall in isotonic medium (1 M mannitol) by digestive enzymes from *Helix pomatia*, spheroplasts (protoplasts) are obtained in which the cytoplasmic membrane is no longer protected by the polysaccharide network of the cell wall. All modifications of ribonuclease were found to destroy the protoplasts very efficiently. The cytoplasmic membrane contains ribonucleic acid, but the cell wall is devoid of this material.

The inefficient action of some modifications of ribonuclease on whole cells is explained, therefore, by the shape and the state of polymerization of the enzyme, which precludes penetration of the pores of the cell wall.

The details of ribonuclease action on yeast cells may be visualized as follows: Adsorption of the enzyme by the phosphoric acid groups of the cell surface takes place. The conformational state of the ribonuclease molecule then determines whether it can penetrate the pores in the cell wall and act on the membrane. Even of the best preparations, only a fraction of the molecules can penetrate; most of the material is adsorbed and incapacitated on the cell wall. The primary step, adsorption on the surface, is impeded by competing cations. The most effective inhibitor, spermine, blocks ribonuclease in a molar ratio 1:1. The inhibitory effect of phosphate is different; it seems to concern the conformation of the enzyme.

The penetrating molecules of ribonuclease cause lesions in the ribonucleic acid component of the cytoplasmic membrane. Profound internal disorganization of the cells indicates that the enzyme gets into the interior.^(3, 4)

CONCLUSIONS

These observations offer auspicious prospects of further exploration of the yeast cell wall and membrane. An opportunity presents itself to use ribonuclease as a tool of differential extraction of yeast, and as a pace-maker for the introduction of substrates, reagents, and other enzymes into the cells.

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STUDIES ON NUCLEAR PROTEINS

A. D. Barton

PURPOSE AND METHODS

These studies on nuclear proteins stem from a long-term interest in the functions of the cell nucleus. Much of the previous work in this project has dealt with a class of soluble proteins that are retained when rat liver cell nuclei are isolated in sucrose media and are liberated when the isolated nuclei are extracted with an ionic medium, such as isotonic saline. These *saline-soluble* proteins of the nucleus are relatively basic compared with the soluble proteins of the cytoplasm, although not so basic as histones. They are retained in the nucleus by ion binding, and their behaviour in moving-boundary electrophoresis changes with changes in the physiological status of the animal.⁽¹⁾ More recently, these studies have been extended to include investigation of other types of nuclear protein under conditions in which the metabolic condition of the animal is altered—during enlargement of liver cell nuclei with thioacetamide administration, for example.⁽²⁾ In the present studies,⁽³⁾ the liver cell nuclei isolated from animals treated with thioacetamide have been extracted with 0.14 *N* NaCl–0.18 *mM* CaCl₂ to obtain the *saline-soluble* proteins as before;⁽²⁾ then with 0.1 *M* phosphate buffer to obtain the *phosphate-soluble* proteins; and then with 0.2 *N* HCl to obtain the *histone* fraction. After extraction of the nucleic acids, the remaining pellet constitutes the *residue* protein fraction. The interesting response of the histone fraction to thioacetamide administration has led to our studying the proteins of this fraction by means of amino acid analysis, chromatography on carboxymethylcellulose, and electrophoresis, both with moving boundary and in acrylamide gels.

PROGRESS REPORT

The experiments with thioacetamide were carried out with the aid of Associated Colleges of the Midwest Semester Student, Ellen C. Anderson;⁽³⁾ the results are presented in Table 87. The administration of thioacetamide produces a decrease in the saline-soluble protein,

no change in the phosphate-soluble protein and substantial increases in the histone and residual protein fractions.

Amino acid analysis indicates that our whole histone fractions are similar to histones in amino acid composition, including an almost complete absence of cystine, but they have a distinctly lower content of basic amino acids.

This indicates the presence of what might be called "nonhistone" protein, in the sense that it contains less than 25% basic amino acid residues. The whole histone fractions behave like histone in giving no precipitate in the HgSO₄–H₂SO₄ test of Mirsky and Pollister. They give patterns typical of recognized histone mixtures in moving boundary electrophoresis, with no obvious slowing or alteration of the components due to the presence of the nonhistone protein. There is no evidence for any significant amount of material with negative mobility,

TABLE 87. CHANGES IN CONSTITUENTS OF RAT LIVER NUCLEI WITH DAILY ADMINISTRATION OF THIOACETAMIDE

Constituent	Period of treatment		
	Control* (n† = 6)	4 days (n = 5)	11 days (n = 5)
Total protein‡/DNA	5.00 ± 0.22§	5.35 ± 0.24	5.90 ± 0.21
Saline-sol. protein/DNA	1.09 ± 0.07	0.89 ± 0.04	0.80 ± 0.03
Phosphate-sol. protein/DNA	0.45 ± 0.02	0.50 ± 0.02	0.46 ± 0.05
Histone/DNA	2.24 ± 0.11	2.58 ± 0.17	3.04 ± 0.14
Residue protein/DNA	1.22 ± 0.04	1.38 ± 0.06	1.60 ± 0.07
RNA/DNA	0.18 ± 0.01	0.29 ± 0.01	0.36 ± 0.02

* Average of determinations on 3 control groups treated with saline for 4 days and 3 control groups treated with saline for 11 days.

† The number of groups analyzed; each group consisted 4 rats.

‡ The sum of the protein fractions.

§ Mean ± standard error of the mean.

either in moving boundary electrophoresis at pH 8.6 or in acrylamide gel electrophoresis at pH 2.3, 4.3, or 9.5.

These histone preparations have been fractionated on carboxymethylcellulose columns, by elution with stepwise increases in salt concentration and then in acid concentration, extending the procedure of Johns *et al.*⁽⁴⁾ The first fractions eluted contain as high as 27% basic amino acid residues and have a ratio of basic to acidic residues as high as 2.5; they resemble the F₁ histones in composition. In general, the content of basic amino acids decreases progressively with successive eluents; the final elution with 0.02 N HCl yielding protein containing approximately 19 percent basic amino acid residues, with a ratio of basic to acidic residues as low as 0.8. The chromatography provides considerable resolution as judged by the different patterns successive fractions may yield in gel electrophoresis. Nevertheless, none of the chromatographic fractions showed any significant amount of material with negative mobility in gel electrophoresis.

CONCLUSIONS

The nonhistone proteins in our histone fractions are of particular interest, because our current results suggest that the increase in the histone fraction that is in-

duced by thioacetamide involves an increase in the proportion of the nonhistone proteins present.

It is possible that these nonhistone proteins are acidic proteins that bind to the histones by salt linkages so firmly that they have successfully resisted all attempts to separate them so far. However, our results are more consistent with the hypothesis that they are actually very basic proteins like the histones, having highly positive electrophoretic mobility, as well as the other properties typical of histones mentioned above. A protein with a low ratio of basic to acidic residues may still be strongly basic if, as in lysozyme for example, many of its distal carboxyl groups are present in the amide form.

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STUDIES ON RAT LIVER RIBONUCLEASES

Y. E. Rahman

PURPOSE AND METHODS

These studies on ribonucleases are related to previous work⁽¹⁾ concerning the lysosome particles in various mammalian tissues; these particles were shown, as a result of experimental findings from several laboratories, to contain twelve acid hydrolases;⁽²⁾ acid ribonuclease is one of them. During the last few years, acid phosphatase has been used most frequently, in some cases exclusively, as the enzyme marker for studies on lysosomes; so it was thought worth while to investigate the lysosomes using some hydrolase other than acid phosphatase. Because of the obvious importance of the role played by ribonucleases in nucleic acid metabolism, these enzymes were chosen as the subject of the present study.

It was reported in 1954 by Roth,⁽³⁾ as well as by Lamirande *et al.*,⁽⁴⁾ that rat liver contained two different ribonucleases, one in the acid and the other in the alkaline range of pH. While the acid ribonuclease seems to be unequivocally localized in the lysosomal

fraction, the intracellular distribution of the alkaline ribonuclease has remained a subject of dispute. The present study was then undertaken with the following purposes in mind: 1) to use the acid ribonuclease as lysosome marker for further studies on the behavior of these particles, 2) to investigate the intracellular distribution of the alkaline ribonuclease, and 3) to study the metabolic functions of these two ribonucleases.

Methods in use are mainly differential and density-gradient centrifugations, and determinations of various lysosomal enzymes.

PROGRESS REPORT

The experimental results confirmed the finding by de Duve *et al.*⁽²⁾ that the intracellular localization of the acid ribonuclease is solely in the lysosomal fraction.

The results of the present study also confirmed the finding of Roth that, in rat liver supernatant, there exists a natural inhibitor for the alkaline ribonuclease.

TABLE 88. COMPARISON OF RAT LIVER RIBONUCLEASES

	RNAase I	RNAase II	RNAase III
pH optimum	5.0	8.0	9.5
Heat	Labile	Resistant	Labile
Freezing or 0°C	Resistant	Resistant	Labile
Acid	Resistant	Resistant	Labile
Cations (mono-, divalent)	Insensitive	Inactivated at high conc.	Inactivated at very low conc.
Inhibitor in supernatant	No	Yes	No
Subcellular distribution	Lyso-somes	Nuclei 5% Microsomes 20% Lysosomes 75%	Intracellular membranes (?)

But this alkaline ribonuclease is not located in the mitochondrial fraction, as reported by the same author.⁽³⁾

The present study has revealed, besides the two previously known ribonucleases, a third ribonuclease which shows an optimum pH at about 9.5. This ribonuclease has different characteristics which indicate that it is a different enzyme from the previous ones.⁽⁵⁾ The summary of various characteristics of all three ribonucleases of rat liver is shown in Table 88.

Rat kidney was found to contain this third ribonu-

lease in the present study, whereas spleen and thymus did not show high activity of this enzyme.

CONCLUSIONS

There are three different ribonucleases in rat liver and kidney. Their intracellular localization is different, although the precise localization of the third ribonuclease found under the present study will require further experimentation. The biological implication of this finding should be of interest for a further understanding of nucleic acid metabolism at the subcellular level.

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THE CONTROL OF RIBOSOME SYNTHESIS IN BACTERIA

Richard E. Ecker

PURPOSE AND METHODS

It was the purpose of this investigation to study the possible role of ribosomal RNA as a controlling factor in the synthesis of ribosomal protein. Two bacterial mutant strains were employed: 1) A strain of *Escherichia coli* which is relaxed^(1, 2) in the control of ribosomal RNA synthesis; 2) A purine-requiring strain of *Salmonella typhimurium*.

Using states of unbalanced growth,⁽³⁾ induced when the required factors were removed, or restored after a period of deprivation, kinetics of synthesis of RNA and protein were studied. Incorporation of valine was used as a measure of the rate of protein synthesis, and uracil incorporation was used to indicate the rate of synthesis of RNA. In addition, some studies with the *E. coli* strain involved the induction and assay of the enzyme β -galactosidase. These studies were made through the use of standard enzymological procedures.

PROGRESS REPORT

The relaxed strain, when deprived of the required amino acid, methionine, continues RNA synthesis in

the absence of concomitant protein synthesis (Figure 148). In an amino acid auxotroph with normal control of RNA synthesis (a *stringent* strain), both RNA and protein synthesis are inhibited when a required amino acid is removed (Figure 149). Similarly, the patterns of synthesis, after restoration of the amino acid, are quite different in the stringent strain as compared to the relaxed strain, in which a large amount of RNA had accumulated during starvation. RNA synthesis increased in rate with very little lag when the tryptophan was restored to the stringent strain, while in the relaxed strain it increased in rate with a significant lag and attained the preshift level some 15 minutes later than the stringent mutant. On the other hand, protein synthesis in the relaxed strain increased to more than half its ultimate rate within the first minute after methionine was restored. When tryptophan was restored to the stringent strain, protein synthesis returned to normal with a half-time of about 20 minutes.

When 6-mercaptopurine (6MP), an inhibitor of nucleic acid synthesis, was added to the relaxed culture just prior to restoration of the methionine, protein syn-

thesis was relatively unaffected, while RNA synthesis decreased markedly (Figure 148).

When the relaxed mutant was induced for the production of β -galactosidase a few minutes prior to deprivation, and the inducer was present in the culture throughout the remainder of the experiment, no messenger RNA (m-RNA) for the production of this enzyme was accumulated during the starvation period; nor was this m-RNA synthesized for some time after the methionine was restored (Figures 150 and 151). Figure 150 shows the growth of the culture before the methionine was removed, during methionine deprivation and after the amino acid was restored; it also indicates the time the inducer, isopropylthiogalactoside

FIG. 148.—Rate of valine and uracil incorporation in unbalanced growth of a relaxed mutant of *E. coli*. Methionine was removed at time = 0 and restored after 62 minutes. Open circles and open triangles refer respectively to valine and uracil incorporation in the presence of 6MP.

FIG. 149.—Rate of valine and uracil incorporation in unbalanced growth of a stringent, tryptophan-requiring strain of *E. coli*. Tryptophan was removed at time = 0 and restored after 61 minutes.

FIG. 150.—Growth of a relaxed mutant before and during methionine deprivation and after methionine restoration.

FIG. 151.—Differential plot of β -galactosidase production as a function of increase in culture density during the experiment shown in Figure 150. Open circles refer to the methionine deprived culture, before and after restoration. Open triangles indicate a culture in which 6MP was added just prior to methionine restoration.

(IPTG), was added. The increase in density during starvation is due primarily to the accumulation of the relaxed RNA. There is a rapid increase in culture density after methionine restoration but it is apparent that enzyme synthesis makes no contribution to this early increase, since, as shown in Figure 151, there is essentially no β -galactosidase synthesis until after the culture has experienced substantial growth.

When the purine-requiring mutant of *S. typhimurium* is deprived of the required factor, little or no RNA or DNA synthesis occurs, while protein synthesis continues for several hours.⁽³⁾ Postshift protein synthesis appears to consist of two components, one synthesized at a constant rate and for an indefinite period of time; the other produced at an exponentially-decaying rate. If

deprivation is preceded by a short pulse of radioactive uracil and the RNA of the cell is subjected at various times to endogenous degradation by incubation in the presence of 2,4-dinitrophenol, the amount of such sensitive RNA in the cell decays, after deprivation, with kinetics identical to those of protein synthesis, that is, both the cellular content of preribosomal RNA and the rate of protein synthesis decay exponentially with time after the shift, and with the same rate constant.

A control model has been proposed, relating the synthesis of ribosomal protein to the cellular content of preribosomal RNA. The assumptions in the model are: 1) the synthesis of ribosomal protein is directed by preribosomal RNA; 2) the rate of protein synthesis is proportional to the cellular concentration of this RNA; and 3) RNA is removed from the preribosomal pool in proportion to the amount of protein synthesized, with the RNA and the newly-synthesized protein combining ultimately to form complete ribosomes. When these assumptions are put in mathematical form and the resulting equations expressed in terms of the kinetics of protein synthesis, the model correctly predicts the observed experimental results.⁽⁴⁾

CONCLUSIONS

The relaxed mutant accumulates an RNA-rich ribosome precursor. It does not accumulate m-RNA for enzyme synthesis, nor does it synthesize enzyme for some time after growth conditions are restored. Yet,

when conditions are restored, it produces ribosomal protein in large quantities.⁽²⁾ Postshift protein synthesis begins much too rapidly to be mediated by m-RNA synthesized after the methionine is restored. Thus, it is concluded that the observed postrestoration synthesis of protein represents the synthesis of ribosomal protein, mediated by the relaxed RNA which had accumulated during methionine starvation.

The purine mutant provides an analogous experimental situation. In this case the preribosomal RNA which is responsible for the postshift synthesis of ribosomal protein is synthesized in normal, balanced growth. Taken together, the two lines of evidence support the suggested model; that is, that preribosomal RNA acts as the messenger for the synthesis of ribosomal protein and then condenses with the newly-synthesized protein to form ribosomes.

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STUDIES ON THE CYTOPLASMIC GRANULES IN *SACCHAROMYCES CEREVISIAE* DURING SPORULATION

Richard E. Alper and George Svihla

PURPOSE AND METHODS

A distinctive feature of the sporulating yeast cell is the presence of numerous cytoplasmic granules.⁽¹⁾ During sporulation, most of the granules disappear. A few are seen inside the spores, but the majority appear to have taken part in the formation of the spore coats. Our purpose was to investigate the nature (e.g., structure, origin, and function) of these granules and the part they play in spore formation. An important aid to the investigation was the alteration of the physiology and morphology of the cells by culture in a medium deficient in nitrogen. Under these conditions, many large cytoplasmic granules are formed, that furnish an abundant supply of material for analysis by gas-liquid and thin-layer chromatography. Ultraviolet microscopy gave some insight into the sporulation process,^(1, 2)

but the greater resolution of electron microscopy was considered necessary for more detail. For these studies, cells were fixed in potassium permanganate and osmium tetroxide and stained with uranyl acetate.

PROGRESS REPORT

Culture of *Saccharomyces cerevisiae* ATCC 7752 in nitrogen-deficient medium resulted not only in the production of cells with numerous large cytoplasmic granules (Figure 152), but also in approximately 50 percent sporulation. This is taken as further evidence of a close relationship of the granules with the sporulation process. When sporulation occurs in cells containing normal size granules (as compared to the large granules of nitrogen-deficient cells), most of the material of the granules is used in formation of the spore coats. In

FIG. 152.—Large lipid granules in the cytoplasm of a vegetative cell after 48 hours in a nitrogen-free medium. There is an absence of well-formed mitochondria at this stage.

FIG. 153.—Spores of *S. cerevisiae* containing lipid granules. The lipid remaining outside the spores is spreading throughout the ascus, coating the spores.

nitrogen-deficient cells, there is more material than is necessary. Ultraviolet microscopy of these cells revealed an ultraviolet-absorbing material between the spores. The material appeared to be holding the spores together by capillary action. It appeared to wet the

outer spore coat. This was confirmed by electron microscopy (Figures 153 and 154).

Analysis of cells grown in the presence of ample nitrogen and under nitrogen-deficient conditions showed a threefold difference in the amount of lipid; the nitro-

FIG. 154.—At higher magnifications the lipid material derived from the cytoplasmic granules can be seen coating the outer spore coat. Note the large granules incorporated into the spore cytoplasm.

gen-deficient cells contain the larger amount. These lipids were composed primarily of ergosterol and a mixture of palmitoleic, oleic, stearic, and palmitic acids.

The cytoplasmic granules that take part in spore formation contain lipids, but what is the origin and structure of the granule and how does it accumulate lipids? We have a few hints. While mitochondria are plentiful in cells grown in a medium with ample nitrogen, very few mitochondria are seen in cells grown in the nitrogen-deficient medium. Instead, these cells are filled with cytoplasmic granules. The number of mitochondria present when cells are grown in the presence of nitrogen is about equal to the number of cytoplasmic granules in nitrogen-deficient cells. When cells grown in the nitrogen-deficient medium, are transferred to the nitrogen-containing medium, mitochondria gradually reappear. This suggests that some mitochondria may become filled with lipid and may become cytoplasmic granules; the process is reversible. What appear to be stages in this transformation have been observed by electron microscopy.

ULTRAVIOLET MICROSCOPY AS A TOOL FOR CYTOLOGICAL AND BIOCHEMICAL RESEARCH

George Svihla, Edward Balish, and Julia L. Dainko

PURPOSE AND METHODS

Ultraviolet microscopy permits the observation of the location and concentration of certain naturally-occurring chemical substances present in the living cell. There are limitations which must be recognized and accepted, or circumvented. Ultraviolet energy can be injurious to living protoplasm, but this effect can be kept at a minimum and may be turned into an advantage for the study of irradiation effects. Another important factor is the size of the subject under investigation related to the concentration of ultraviolet-absorbing material it contains. We cannot adjust the concentration of samples for optimum measurement, as is usually done in spectrophotometry. Concentrations present in the living organism are dictated by the state of life itself. Specimens for UV studies, therefore, must be carefully selected.

The yeast cell is a good example. The content of its ultraviolet-absorbing materials lies within the range required for effective observation by ultraviolet microscopy, and its lack of motility is an important factor in the production of useful images with the available ultraviolet energy.

The morphological and physiological changes that occur in yeasts under varied culture conditions, coupled

CONCLUSIONS

Under nitrogen-deficient conditions, the normal metabolism of *S. cerevisiae* is altered so that lipids accumulate in cytoplasmic granules that appear to have been mitochondria. These cytoplasmic granules take part in spore formation, where they serve as a possible source of structural and nutritive materials. Whether these materials are used directly or are modified was not determined. Under suitable conditions, the lipid in the cytoplasmic granules can be metabolized, and apparently the organelles that result are mitochondria.

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with the rather unique advantages of an ultraviolet microscope that provides rapid access to high-quality, photographic images, is the basis for this program. It is the purpose of this program to exploit these combined opportunities in the conduct of unique cytological research.

Morphological changes and changes in the location and concentration of ultraviolet-absorbing materials in the living cell are observed by ultraviolet microscopy. These results are supported by chemical analyses, chromatography, spectrophotometry, and other techniques on comparatively large samples.

PROGRESS REPORT

The importance of S-adenosylmethionine in the biosynthesis of numerous biological compounds, including phospholipids and sterols, led us to search for a common denominator for the production and accumulation of this compound in yeasts and related organisms. During 1965, numerous yeasts and molds, grown under conditions suitable for the production of S-adenosylmethionine and its accumulation in the vacuole, were examined by ultraviolet microscopy.^(1, 2) The following yeasts, *Hansenula anomala*, *Pichia fermentans*, *Pichia membranaefaciens*, *Saccharomyces ludwigii*, and *Can-*

FIG. 155.—Photomicrographs of several Fungi Imperfecti obtained using ultraviolet energy of $265\text{ m}\mu$ wavelength to illustrate the localization of S-adenosylmethionine in the organisms when they are grown under conditions that lead to accumulation of an unusual amount of the sulfonium compound: a) *Hansenula anomala* containing $18\text{ }\mu\text{M/g}$ of cells; S-adenosylmethionine is present in greatest concentration in the darkest vacuoles; b) *Pichia membranaefaciens* containing $11\text{ }\mu\text{M/g}$; c) *Saccharomyces ludwigii*, $13\text{ }\mu\text{M/g}$; d) *Ustilago maydis*, $6.5\text{ }\mu\text{M/g}$.

didia albicans, were found to produce and accumulate S-adenosylmethionine. The quantities of the sulfonium compound produced were not as large as is typically obtained with *Candida utilis* and *Saccharomyces cerevisiae*, however.

The morphology of *H. anomala* (Figure 155a), *P. fermentans*, *P. membranaefaciens* (Figure 155b), and *C. albicans* is quite similar to that of *C. utilis*; a large vacuole surrounded by cytoplasm that contains numerous mitochondria and one, or occasionally two, lipid bodies (cytoplasmic granules) is characteristic. *Saccharomyces ludwigii* (Figure 155c) and *S. cerevisiae* differ from these species by the possession of large numbers of lipid bodies. The lipid bodies have been of interest in relation to spore formation in *S. cerevisiae*,

where they take part in formation of the spore coat. Equally important is the close association of lipids with membranes in general. The lipid bodies appear to be derived from mitochondria and to revert to mitochondria as the lipid is metabolized under suitable conditions.⁽³⁻⁵⁾

Large lipid bodies are produced when these yeasts are grown under nitrogen-deficient conditions (*S. cerevisiae* illustrated in Reference 5). Cells that contain large lipid bodies have little S-adenosylmethionine and few, or no, mitochondria. Cells that contain large amounts of S-adenosylmethionine have few, or small, lipid bodies, and appear to have numerous mitochondria by UV microscopy. Therefore, one might infer that in these yeasts, under the special culture conditions im-

posed, S-adenosylmethionine is produced by the mitochondria and accumulated in or by the vacuole.

In this study, similarities and differences have been sought in the production of S-adenosylmethionine at the genus and species level. The ability of *C. albicans* to exist in different forms (yeast, pseudomycelial, and mycelial), relative to culture conditions or mutant strain, offers an opportunity to observe S-adenosylmethionine accumulation in variants of the same species. Cultures of *C. albicans* ATCC 10231 in supplemented medium yielded 5 μ moles of S-adenosylmethionine per gram of cells; this is about one-fourth the quantity usually obtained with *C. utilis*. Lipid bodies were prominent in these cells. The same was true of *C. albicans* ATCC 10261. The mycelial form, ATCC 10259 (derived from 10261), grown in supplemented medium, yielded cells with smaller lipid bodies and a concentration of 15 μ moles/g, while cells grown in non-supplemented medium produced only 5 μ moles/g; the latter is unusual because it is five times greater than the amount obtained with nonsupplemented *C. utilis*. The observations on the mycelial form of *C. albicans* were also of interest because of their similarity to the morphology of molds. A survey of numerous molds, grown under various culture conditions has never resulted in levels of S-adenosylmethionine greater than 2 μ moles/g of mycelium.

THE LIPIDS OF ENTEROBACTER CLOACAE

Nora Larson

PURPOSE AND METHODS

Interest in bacterial lipids⁽¹⁾ has accelerated since Hofmann's⁽²⁾ discovery of lactobacillic acid in *Lactobacillus arabinosus*. Development of effective techniques has made studies in this area more fruitful and has led to greater expenditure of effort to characterize the nature of fats occurring in bacteria. The demonstrated ability to *Enterobacter cloacae* to synthesize fats⁽³⁾ and the availability of new techniques for lipid analysis⁽³⁻⁵⁾ have provided the basis for the study of this bacterium.

The organism, a strain of *Enterobacter cloacae* American Type Culture Collection No. 961, is a gram-negative bacterium distinguished by the presence of flagella. Large yields have been obtained by growing the bacteria in 10-liter amounts of medium, vigorously aerated, at a temperature of 36°C for three days. The medium consists of 2% peptone, 3% glucose, and 3% glycerol in water. Extraction of these ingredients yields less than 10 mg of fatty acids per liter. Wet cells have

CONCLUSIONS

General rules that predict the formation of S-adenosylmethionine in various yeasts, have not yet been found. A high rate of metabolism does seem to be a prerequisite, however. In all species that have large amounts of the sulfonium compound, a well-developed vacuole is present, but possession of vacuoles, in itself, does not assure high levels of these compounds. When the energy resources of the cell (ATP level) divert into other metabolic channels, such as the formation of cytoplasmic lipids, the concentration of S-adenosylmethionine remains low.

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been saponified in aqueous ethanolic potassium hydroxide and the crude fatty acids extracted and esterified with diazomethane.⁽⁶⁾

PROGRESS REPORT

The yield of fatty acids is approximately 10% of the dry weight of the cells. Gas-liquid chromatography of several batches indicates the presence of two unusual fatty acids. These have been identified as cyclopropane acids by selective hydrogenation and verified by infrared spectroscopy, which shows the characteristic absorption band at 1020 cm^{-1} . The structures have been characterized as 9,10-methylenehexadecanoic acid, and 11,12-methyleneoctadecanoic (lactobacillic) acid. The amount of cyclopropane acids has been estimated from the infrared spectrum to be about 30% of the fatty acids present, which is consistent with quantity found by triangulation of the recorder peaks from gas-liquid chromatography. Monoenoic acids of C-14, C-16, and C-18 chain lengths are presented as

well as saturated acids, C-12, C-14, C-16, and C-18, of which C-16 comprises 32.6% of the total.

Whole cells and cells subjected to manipulation in a French press have been extracted by the method of Folch *et al.*⁽⁷⁾ The presence of phospholipids has been demonstrated by thin-layer chromatography. The dominant fraction appears to be phosphatidyl ethanolamine. Work is in progress to identify other lipid components and to obtain quantitative data. The analyses will be extended to additional members of the species *Enterobacter cloacae*.

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Isotope Studies: Techniques and Applications

EFFECT OF DEUTERIUM OXIDE ON PANCREATIC LIPASE

John F. Thomson, Karen J. Bush,* and Sharron L. Nance

PURPOSE AND METHODS

In most of the studies on enzymatic activity in D_2O , soluble substrates have been employed. However, there are a large number of enzymes that act on insoluble substrates, for which the conventional Michaelis-Menten kinetic analysis is inappropriate.⁽¹⁾ One of these is pancreatic lipase, shown by Sarda and Desnuelle⁽²⁾ to act only on emulsified esters; although crude preparations could hydrolyze water-soluble compounds such as triacetin, the highly purified enzyme had almost no effect on this substrate until it was present in excess of its solubility.

Thus pancreatic lipase seemed to be an interesting enzyme on which to study solvent effects of deuterium oxide. Apparently the only previous work with this system was carried out in 1934 with an impure preparation;⁽³⁾ hydrolysis of triolein was not affected by 30% D_2O .

Crude porcine pancreatic lipase (Sigma Chemical Co.) was purified by the method of Baskys *et al.*⁽⁴⁾ Paper electrophoresis of the final product revealed one major and one rather diffuse minor component. Although the dried enzyme appeared to be stable when stored in the cold, aqueous solutions deteriorated rather rapidly; addition of glycerol, cysteine, or deoxycholate did not prevent the loss of activity. Each

sample of enzyme was weighed and dissolved in water. The solution was divided into a number of 0.5-

or 1.0-ml samples which were frozen until immediately before use.

The enzyme was active against glycerol triesters of

FIG. 156.—Optimum pH and pD for purified pancreatic lipase. Reaction mixtures contained 0.91 millimoles of emulsified tributyrin in 10 ml.

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FIG. 157.—Hydrolysis of tributyrin in H_2O (triangles) and D_2O (circles). Reaction mixtures contained 0.91 millimoles of substrate in 10 ml.

acetic, propionic, butyric, and oleic acids. Hydrolysis of triolein was very slow unless sodium deoxycholate was added to the reaction mixture.^(2, 4)

Tributyrin was used as the substrate in most assays. Emulsions were prepared in 10% gum arabic according to Sarda and Desnuelle;⁽²⁾ generally, 3 ml of tributyrin were blended with 20 ml gum arabic. The number of particles per unit volume was counted in a Coulter counter. The total number of particles and their distribution appeared to be identical in both H_2O and D_2O . Other emulsifying agents (Triton X-100 and BRIJ-98) proved to be unsatisfactory—at the concentrations necessary to stabilize the emulsions, they inhibited the enzyme.

Lipase activity was measured titrimetrically. An automatic titration apparatus⁽⁵⁾ was converted to a pH-stat so that the acid formed by hydrolysis of the tributyrin was continuously neutralized to the predetermined pH. With 0.005 *N* NaOH as the titrant, the fluctuation in pH was about 0.1 unit in the course of an assay. In the experiments with D_2O as solvent, 0.005 *N* NaOD in 99% D_2O was used for titration to avoid dilution of the solvent with H_2O . The rate of hydrolysis was linear for at least 15 minutes under the conditions employed. All experiments were carried out at 25°C.

In most assays, a quantity of the tributyrin emulsion

in gum arabic (0.2 to 5.0 ml) was introduced into the reaction vessel of the pH-stat and diluted with water (or D_2O) to 10 ml. The pH was adjusted to the predetermined value; then the reaction was started by the addition of 0.1 to 0.5 ml of the enzyme solution. The amount of NaOH added was noted at one-minute intervals for 15 minutes. The slope of the plot of volume of NaOH against time was calculated by the method of least squares. The results are expressed as micro-equivalents of NaOH added per minute.

PROGRESS REPORT

pH and pD Optima

Figure 156 shows that in H_2O the pII for optimum lipase activity is about 7.9; in D_2O , the optimum pD is about 8.2.

Effect of Deuterium Oxide

The influence of 99.6% D_2O as solvent is shown in Figure 157. At pH or pD 8.0, near the optima for both solvents, lipase activity was reduced by D_2O to about 46% of control activity. Data at pH 7.5 and pD 8.5 are shown on the same figure.

The effect of lower concentrations of D_2O was studied at four different substrate concentrations (Figure 158). At all four concentrations (0.09, 0.22, 0.90, and 2.24 millimoles tributyrin) there was little inhibition at concentrations of D_2O up to 50%. Lipase activity was decreased by about $\frac{1}{3}$ in pure D_2O at the two

FIG. 158.—Effect of varying concentrations of D_2O on hydrolysis of tributyrin at four levels of substrate: 0.09 (○), 0.22 (△), 0.90 (□), and 2.24 (▽) millimoles in 10 ml.

lowest substrate concentrations, but by about $\frac{1}{2}$ at the two highest concentrations.

CONCLUSIONS

There are as yet insufficient data available to analyze the kinetics of the reaction. However, it would appear from the fact that the rate of hydrolysis increases linearly with increasing enzyme concentration (Figure 157), rather than as a function of the $\frac{2}{3}$ power of the concentration, that the kinetics of pancreatic lipase probably do not satisfy the Gyani-Freundlich isotherm as applied by McLaren.⁽¹⁾

It is clear that pure D₂O has an appreciable inhibiting action; however, at lower concentrations of D₂O the inhibition is nearly negligible. These observations with tributyrin confirm those of Hughes *et al.*⁽³⁾ with triolein.

We are indebted to William J. Eisler for modification

of the pH-stat, to Herbert E. Kubitschek for counting the particles in the emulsion, and to Joseph J. Katz and Henry L. Crespi (Chemistry Division) for supplies of 99.8% D₂O.

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STUDIES ON ACONITASE, WITH PARTICULAR REFERENCE TO ITS ACTIVITY IN D₂O

John F. Thomson, Sharron L. Nance, and Karen J. Bush*

PURPOSE AND METHODS

Several years ago, it was shown that the enzymatic hydration of fumarate in D₂O took place at less than half the rate observed in ordinary water.⁽¹⁾ The reverse step of the fumarase reaction, the dehydration of malate, was inhibited by only 20% in D₂O. There was a slight effect on the apparent equilibrium constant.

This report is concerned with a similar study on another enzyme concerned with reversible hydrations, namely aconitase, which catalyzes the interconversions among *cis*-aconitate, citrate, and α L₅ - β D₅(+)isocitrate (referred to hereafter as L(+)isocitrate).

Speyer and Dickman,⁽²⁾ studying 4 of the 6 interconversions in 93% D₂O, observed inhibitions of 35 to 60%. However they did not measure the effects on maximum velocities or Michaelis constants.

Enzyme

Aconitase prepared from beef heart was used in the preliminary experiments. Most of the kinetic data reported here were obtained on preparations from beef liver. The purification procedures consisted of two ethanol fractionations and one ammonium sulfate fractionation.⁽³⁾ Further purification was not attempted;

after dialysis of the enzyme, it must be reactivated with cysteine and ferrous ion, and the Fe⁺⁺-citrate and Fe⁺⁺-isocitrate complexes interfere with the spectrophotometric estimation of aconitate.

Substrates

Solutions of *cis*-aconitate were prepared from the anhydride. L(+)-3-deuteroisocitrate and 2-deuteroisocitrate were prepared enzymatically from *cis*-aconitate in D₂O; after equilibrium was reached, the reaction was stopped by heating, and the acids were separated by silica gel chromatography. A sample of L(+)-isocitrate was prepared similarly, although in most experiments DL-isocitrate was used.

Analytical Methods

Aconitate was measured by its ultraviolet absorption; 240 m μ was the wave length most frequently used. Citrate was measured by a chemical method⁽⁴⁾ in earlier experiments. Subsequently, we used an enzymatic method involving the enzyme citratase, obtained from *Aerobacter aerogenes* grown in a medium containing citrate.⁽⁵⁾ Isocitrate was also measured enzymatically with isocitric dehydrogenase and TPN.⁽⁶⁾

Assay Procedure

Reaction mixtures consisted of 1.0 ml 0.1 M tris buffer, varying concentrations of one of the three sub-

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FIG. 159.—Effect of ionic strength and pH on aconitase activity. Each reaction mixture contained 0.02 *M* glycine and 0.0013 *M* *cis*-aconitate. The enzyme was prepared from beef heart.

strates, and 0.1 of appropriately diluted aconitase solution in 3 ml total volume. All experiments were carried out at 25°C. The formation or disappearance of aconitate was measured by following the change in UV absorption at 240 m μ on a recording chart attached to a Beckman DU spectrophotometer. In many experiments, after a short observation period (usually 3 minutes) the reaction was stopped and the mixture was analyzed for the three acids.

Kinetic Analysis

Measurements were made, in both H₂O and D₂O, of the Michaelis constants of aconitate, citrate, deuterocitrate, DL-isocitrate, L(+)-isocitrate, and L(+)-deuteroisocitrate, and of the maximum velocities for each possible interconversion among the substrates.

PROGRESS REPORT

Optimum pH and Effects of Ionic Strength

Morrison showed that aconitase activity was profoundly influenced by the buffer system.⁽⁷⁾ We have confirmed and extended his observations, and have shown that the ionic strength of the assay system is of considerable importance. Figure 159 illustrates this point. With 0.02 *M* glycine buffer, the optimum pH in the absence of sodium chloride was about 7.0. In the presence of 0.2 *M* NaCl, however, there was marked inhibition of activity below pH 6.5, but stimulation

above pH 7; and the optimum pH was shifted from 7 to 8.

Somewhat different effects of sodium chloride were noted with other buffers. Since the pH optima for the three substrates are somewhat different, and since the activation effect of increased ionic strength was variable, we elected to carry out all assays in 0.033 *M* tris buffer, pH 7.4, with no electrolyte added. Assays in D₂O were carried out at pD 7.4.

Deuterium Effects on Kinetics

Values for the Michaelis constants and maximum velocities are listed in Table 89. It is apparent that there was considerable variation in the degree of inhibition of the various interconversions. One of the most striking effects was seen in the relative amounts of citrate and isocitrate formed from aconitate in D₂O. In ordinary water, the initial rates of formation of citrate and isocitrate are approximately the same;

TABLE 89. EFFECT OF D₂O ON MICHAELIS CONSTANTS AND MAXIMUM VELOCITIES OF SUBSTRATES FOR ACONITASE

	H ₂ O	D ₂ O	D ₂ O/H ₂ O ratio
<i>cis</i> -Aconitate			
V_{-A}^*	1.00	0.46	0.46
V_{AC}	0.53	0.14	0.26
V_{AI}	0.47	0.32	0.68
K_M	$0.99 \times 10^{-4} M$	$0.78 \times 10^{-4} M$	0.79
Citrate			
V_{-C}	0.53	0.16	0.32
V_{CA}	0.25	0.09	0.39
V_{CI}	0.28	0.07	0.26
K_M	$0.95 \times 10^{-3} M$	$0.85 \times 10^{-3} M$	0.89
2-Deuterocitrate			
V_{-C}	0.51	0.15	0.30
V_{CA}	0.24	0.09	0.36
V_{CI}	0.27	0.06	0.24
K_M	$1.00 \times 10^{-3} M$	$0.94 \times 10^{-3} M$	0.94
L(+)-Isocitrate			
V_{-I}	0.85	—	—
V_{IA}	0.41	—	—
V_{IC}	0.44	—	—
K_M	$1.39 \times 10^{-4} M$	—	—
L(+)-3-Deuteroisocitrate			
V_{-I}	0.81	0.22	0.27
V_{IA}	0.39	0.115	0.29
V_{IC}	0.42	0.105	0.25
K_M	$1.27 \times 10^{-4} M$	$1.04 \times 10^{-4} M$	0.82
DL-Isocitrate			
V_{-I}	0.82	0.22	0.26
V_{IA}	0.39	0.11	0.28
V_{IC}	0.43	0.11	0.25
K_M	$1.48 \times 10^{-4} M$	$1.34 \times 10^{-4} M$	0.90

* V_{-A} is the maximum velocity for disappearance of *cis*-aconitate; V_{AC} and V_{AI} are maximum velocities of formation of citrate and isocitrate, respectively, from aconitate. Maximum velocities are given in arbitrary units; all values are relative to $V_{-A} = 1$.

TABLE 90. PERCENTAGE COMPOSITION OF ACONITASE
EQUILIBRIUM MIXTURE IN H₂O AND D₂O

	H ₂ O		D ₂ O	
	Observed*	Calculated†	Observed‡	Calculated§
Citrate	88.1 ± 1.3	97.7	86.7 ± 2.3	88.0
Isocitrate	7.5 ± 1.0	7.8	8.1 ± 1.2	9.0
Aconitate	4.1 ± 0.2	4.6	5.1 ± 1.3	3.0

* Average of 9 measurements with standard deviations.

† Calculated from data in Table 89 for *cis*-aconitate, citrate, and L(+)-isocitrate in H₂O.

‡ Average of 6 measurements with standard deviations.

§ Calculated from data in Table 89 for *cis*-aconitate, 2-deuterocitrate, and 3-deuteroisocitrate in D₂O.

$$\frac{[A]}{[C]} = \frac{K_A}{K_C} \left(\frac{V_{CA}V_{IA} + V_{CA}V_{IC} + V_{IA}V_{CI}}{V_{AC}V_{IA} + V_{AC}V_{IC} + V_{AI}V_{IC}} \right)$$

The agreement between the calculated and observed results in H₂O are excellent. In D₂O, however, the percentage of aconitate predicted from the kinetic data is too low, although the ratio of citrate to isocitrate agrees fairly well with the observed value. The source of the discrepancy is probably the value for the Michaelis constant for aconitate in D₂O, for which a large standard error (nearly 50% of the value) was observed. If only the two highest numbers of the eight individual measurements are used, K_m for aconitate becomes 1.1×10^{-4} M, and the equilibrium concentration for aconitate rises to 4.2%.

FIG. 160.—Coupled assay of aconitase and citratase. Each reaction mixture contained 0.033 M tris buffer, pH 7.4, 0.003 M MgSO₄, 0.0016 M DL-isocitrate, 7.6×10^{-5} M NADH, and 0.1 ml citratase solution. The reaction was started by adding the indicated amount of beef-heart aconitase solution. (Redrawn from chart tracings at 340 mμ.)

however, in D₂O the rate of formation of isocitrate is approximately double that of citrate.

The Michaelis constants for all three substrates were appreciably lower in D₂O than in H₂O. It can be seen that in a given solvent the differences in both the Michaelis constants and the maximum velocities among DL-isocitrate, L(+)-isocitrate, and L(+)-deuteroisocitrate (and between citrate and 2-deuterocitrate) were very slight. The unnatural isomer of isocitrate would seem to be a rather feeble competitive inhibitor.

Deuterium Effects on Equilibrium

In several experiments, the incubations were carried out until equilibrium was reached. The composition of the equilibrium mixture was then determined. Table 90 shows that the principal difference between equilibrium conditions in H₂O and those in D₂O was a slightly higher percentage of isocitrate in D₂O.

The predicted values were calculated from the data in Table 89 according to the following equations:

$$\frac{[I]}{[C]} = \frac{K_I}{K_C} \left(\frac{V_{CI}V_{AC} + V_{CI}V_{AI} + V_{CA}V_{AI}}{V_{AC}V_{IA} + V_{AC}V_{IC} + V_{AI}V_{IC}} \right)$$

Coupled Assays

Among the mechanisms which have been proposed for aconitase are the "linear", "tricyclic", and "centrosymmetric".⁽⁸⁾ According to the linear model, *cis*-aconitate is an obligatory intermediate between citrate and isocitrate, whereas the other two models postulate an interconversion between the aconitase-citrate and aconitase-isocitrate complexes without the formation of free aconitate. If the linear mechanism is correct, there should be a lag period in the formation of citrate from isocitrate (or vice versa). Thus if one prepared for incubation mixture of isocitrate, citratase, malic dehydrogenase Mg⁺⁺, and NADH,* a delay in the reduction of NADH (measured at 340 mμ) after addi-

* The reaction sequence is:

The citratase preparation contained sufficient NDH for the final step to proceed at a rapid rate.

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METABOLISM AND FATE OF TRITIATED THYMIDINE IN THE MOUSE

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PURPOSE AND METHODS

In recent years, tritiated precursors and especially tritiated thymidine have been extensively used in studies to determine the important parameters of the life cycle of the mammalian cell. Most of these studies were performed by high-resolution autoradiography as applied to *in vivo* studies of mammals. Autoradiography of tissues labeled with tritiated thymidine offers the possibility of determining the number of cells in DNA synthesis at the time the radioisotope was injected, which gives an approximate measure of the proliferating activity of tissue. In addition, the fate of the labeled cells can be followed for several life cycles, sometimes until death of the labeled cell. A limitation of autoradiography is that the technique is more qualitative than quantitative. Although methods have been described for a more quantitative determination of autoradiographic findings,⁽¹⁾ the procedure permits only the tracing of the radioactivity incorporated into DNA. It does not give any indication of the fate of tritiated thymidine injected into the animal, but not incorporated into DNA, and of its quantitative distribution in various organs. Such knowledge is of some interest in long-term retention studies, especially those in which the toxicity of tritiated thymidine is investigated.⁽²⁾ This communication is concerned with additional studies and results of our continuing investigations on the fate of tritiated thymidine injected into mice and its distribution into the DNA of various organs and tissues. The incorporation of thymidine was determined, in these studies, by isolating the nucleic acid fractions and measuring the specific activity of DNA. The radioactivity was measured in a Packard Tri-Carb spectrometer after a flash combustion method was used for the assay of the biological samples.⁽³⁾

PROGRESS REPORT

Influence of the Amount of Tritiated Thymidine Injected on the Amount of Radioactivity Incorporated into DNA

The distribution of radioactivity in the acid-soluble and DNA fractions of the whole mouse one hour after

an intraperitoneal injection of tritiated thymidine was measured at doses between 30 μ Ci and 100 μ Ci; the amount of radioactivity incorporated into DNA of mouse tissues varies between 8% and 9% of the injected dose. More than 90% of the radioactivity injected is found in the acid-soluble fraction (ASF), where about two thirds of it is in the form of tritiated water. The remaining radioactivity found in the ASF is probably due to thymine which is rapidly produced by cells exposed to exogenous thymidine. At less than 30 μ Ci per mouse, the amount of radioactivity incorporated into DNA of the intact mouse is only 6% of the injected dose. This is paralleled by an increase in the percentage of tritiated water in the ASF. At 100 μ Ci per mouse, there is, however, a sharp decline in the fraction of radioactivity found in tritiated water.

In the small intestine and remainder of the animal the amount of radioactivity incorporated into DNA increases in proportion to the dose injected, that is, the fraction of injected radioactivity incorporated into DNA remains approximately constant. In the liver, however, the amount incorporated into DNA reaches a maximum at about 50 μ Ci per mouse and then levels off.

Influence of Age on Distribution of Radioactivity in DNA of Mouse Tissues

The subcutaneous route of injection was chosen for this experiment because intraperitoneal injection in the newborn was found to be unreliable. The amount of radioactivity in DNA of the spleen and gastrointestinal tract and in DNA of the remainder of the animal was determined in male and female mice of the following ages: 4 days, 1 month, 4 months, and 1 year. At 4 days of age, the spleen and gastrointestinal tract together accounted for only 10% of the total radioactivity incorporated into the DNA of the mouse. In 1- and 4-month-old mice, the amount of DNA-bound radioactivity in the spleen and gastrointestinal tract together was 41% of the total, and in 12-month-old mice, it increased to 54% and 58% of the total.

CONCLUSIONS

Intraperitoneal injection of tritiated thymidine in doses ranging from 10 μ Ci to 100 μ Ci per adult mouse resulted in 8% of the injected dose being incorporated into DNA. The remainder of the tritiated thymidine was catabolized primarily to tritiated water. The age of the animal influenced the percentage of thymidine incorporated into DNA of various organs. In adult mice injected intraperitoneally the gastrointestinal tract contained approximately 55% of the total radioactivity incorporated into DNA. At a dose level of 100 μ Ci, this means that one hour after administration of thymidine, the gastrointestinal tract contains about 5 μ Ci of radioactivity. Because, 48 hours later, about half of this radioactivity is shed into the intestinal lumen by epithelial cells reaching the tips of the villi and because

thymidine can be absorbed through the gastrointestinal tract, it is not surprising that radioactivity released from the DNA of dying epithelial cells can be detected in other cells by radiochemical and radioautographic techniques.⁽⁴⁾

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EXCHANGE LABELING CHROMATOGRAPHY ON TRITIATED ALUMINA

Peter D. Klein, John C. Knight, and Eric H. Erenrich

PURPOSE AND METHODS

Chromatographic systems offer many significant advantages in the promotion of isotopic exchange reactions: those of speed, economy, efficiency and a high purity of the labeled compound obtained.⁽¹⁾ Because of obvious applications to the study of intermediates in sterol metabolism, we have been interested in the replacement of enolic hydrogens in ketosteroids during their passage through a basic alumina column previously deactivated with tritiated water.⁽²⁾

PROGRESS REPORT

The following procedural objectives have been attained:

1) The tritium incorporated into ketosteroids during chromatography has been shown to be unaffected by recrystallization in hydroxylic solvents, but 95% can be back-exchanged under strongly basic conditions.

2) The labeled ketosteroids can be reduced to the corresponding alcohols without loss of activity, resulting in a labeled product in which the tritium is completely resistant to further exchange.

3) The hydroxyl surface of the alumina has been exchanged with tritiated water by repeated vacuum drying at elevated temperatures followed by deactivation with liquid water until the surface attained the specific activity of the original water.

4) Model ketosteroids have been shown to undergo complete exchange of all four enolic hydrogens about the keto group. This enables a direct measurement to

be made of the surface specific activity of any labeled alumina by chromatographing a known weight of reference ketosteroid and counting the eluted steroid. The specific activity of the steroid is then four times the specific activity of the alumina —OH group.

5) A linear relationship between the weight of steroid applied and the counts eluted has been obtained for samples from 0.003 to 10.0 mg, thus permitting quantitation of ketone concentration in an unknown sample.

6) Alumina deactivated with tritiated water can be diluted with unlabeled alumina of comparable activity to obtain a chromatographic adsorbent whose activity is reduced in stoichiometric proportions.

7) Nineteen ketosteroid structures have been tested to determine if other enolic hydrogens can undergo exchange. The Δ^4 bond has been shown to influence the orientation of the steroid on the alumina surface such that the 2 position is unable to equilibrate with the surface hydroxyls. All other structures show complete equilibration if conjugation of double bonds during the enolization process does not pass through an alpha carbon with no hydrogens on it.

8) The tritiated alumina has been shown to possess the anticipated ability to resolve ketosteroids as well as to label them in the process. This suggests that a self-labeling chromatographic analysis is feasible.

CONCLUSIONS

Production of labeled ketosteroids with specific activities of 5-10 mCi/mM has been demonstrated to be

easily attainable by exchange labeling chromatography; alternatively the detection of submicrogram quantities of specific ketosteroids is readily achieved. Alumina columns are stable and with reasonable care can be used repeatedly. This reduces the per gram cost of the tritiated adsorbent to economically attractive levels.

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ISOTOPIC FRACTIONATION DURING ANALYTICAL SEPARATIONS

Peter D. Klein and Patricia A. Szczepanik

PURPOSE AND METHODS

This project is concerned with the verification, computation, and measurement of isotopic fractionation phenomena found to occur during the analytical separation and purification of heteroatomic molecules. It utilizes dual-tracer methodology developed in this Division (including liquid scintillation counting) as well as a complex of computer programs which permit solution of the basic equations governing isotopic fractionation from our own data as well as those from other laboratories.

PROGRESS

During the past year an extensive compilation and review of the occurrence of isotopic fractionation has been carried out.⁽¹⁾ Many instances of unexpected or unintentional fractionation during the separation of classes of compounds were examined, and through the courtesy of the authors reporting these incidents, the original data were made available for recomputation in a standard format. Authentic instances were found in a wide variety of separation processes, from distillation to thin-layer chromatography, involving structures ranging from hydrocarbons to carbohydrates and totalling, in all, more than 50 compounds. The resulting overview has made clear several aspects of the importance and future of these measurements. First, the surprisingly frequent encounters with fractionation in tracer methodology indicate that an awareness of this possibility is essential if valid measurements of identity, concentration, and purity are to be made using isotopic tracers. Second, the fractionation factors found in these molecules of molecular weight 200-500 are often as large as those in smaller molecules, thus offering the opportunity to employ differential locations the isotope and to find those locations that either maximize or minimize the isotopic effects. Third, fractionation phenomena may be used to determine exceedingly small thermodynamic quantities. ΔH values

have been reported for heats of mixing in which the isotopic effect was as small as 9.6 cal/mol; ion exchange isotope effects of 0.41 cal/mol have also been determined. In adsorption chromatography, the intramolecular contribution of one portion of the molecule as opposed to another can then be investigated.

It has also been evident that we are moving beyond the "sighting" stage to the utilization of these fractionation factors as research tools to study the separation processes themselves. One of the most interesting findings proved to be the suggestion that these factors could be altered experimentally by parameters accessible to the investigator. A collaborative study with Dr. V. Cejka of the University of Amsterdam has measured the changes in isotopic fractionation of cortisone-1,2- H^3 from cortisone-4- C^{14} during partition chromatography with various stationary phases. Cejka has shown that the methanol concentration of the stationary phase has a profound influence on the displacement of H^3 from C^{14} during the chromatographic process, and he has altered this displacement over a range from 0.3 to more than 6%. The effect shows a maximum in the region of 10% methanol in the stationary phase and reaches a minimal value at a methanol concentration of 80%. The fractionation appears to be made up of two isotope-sensitive processes; the first, a true partition effect in which the H^3 -cortisone migrates perceptibly more slowly than the C^{14} -cortisone and the second, an associative process occurring during the initial introduction of the sample on the column in which some form of aggregate (e.g., micelle) is being formed. The consequence of the second process is to produce an initial fractionation of isotopes which is far greater than the partition process, but as the sample migrates down the column, the associative structures are disrupted or dispersed and the fractionation effect diminishes. This can be shown by comparing the separation factors on short and long columns: on an 8.5 cm column, the difference in migration was 2.6%, whereas on a similar column of

length 28.5 cm, the difference was 0.65%, a fourfold reduction.

CONCLUSIONS

The utility of these findings lies in the minimization of isotope effects (in those instances where its presence is detrimental, e.g., in isotope dilutions studies) or the exacerbation which might be possible. Should it prove possible to enhance the effect to the extent where the difference in mobility is 10–20% (and this is not

excluded by thermodynamic considerations), even moderate efficiency columns of 500–1000 plates would serve to enrich low-level samples. Alternatively, an analytical resolution of stable isotope-containing molecules could be achieved without requiring the use of a mass spectrometer to assay isotope content.

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PERFORMANCE STANDARDS FOR LIQUID SCINTILLATION COUNTERS AND THEIR EVALUATION IN CURRENT MODELS

Peter D. Klein and William J. Eisler

PURPOSE AND METHODS

At the present time, bid specifications and instrument-to-instrument comparisons of liquid scintillation counters are limited by the small number of performance standards which have been generally recognized and accepted. Moreover, most of these standards are applicable or suitable only for single-isotope measurements; examples of these are the expressions of efficiency squared, divided by the background count (E^2/B), or of stability as the percent variation in a standard count over a given interval of time. The growing use of dual-isotope measurements requires the formulation of new standards which accurately (and unambiguously) reflect the inherent capability of an instrument to detect and measure both forms of radioactivity. These standards are concerned with the separation of the two spectra as well as the efficiency with which each isotope is detected and with the stability of isotope ratio values over short-term as well as long-term periods.

Although it has been theoretically possible to establish such criteria before now, the vast amount of hand computation required has deterred investigators from doing so. The availability of computer programs and data transcription facilities for the liquid-scintillation counting system currently in use in this Division presents the opportunity to conduct initial surveys and, on the basis of these results, to reduce the amount of work required to establish the performance characteristics of other commercial models in current production.

PROGRESS REPORT

A study of the recommended procedures for operating liquid scintillation counters showed that

these procedures took into account only the separation of the less energetic isotope from the more energetic isotope; this was achieved by screening out the lower energy isotope spectrum in the second channel. These procedures neglect the contribution of the higher energy isotope to the channel in which the less energetic isotope is counted, and do not appear to have considered the increased separation capability possible when this contribution is reduced. An analysis of the channel-ratio technique for computing isotope ratios has provided the basis for expressing the spectral separation characteristics of an instrument in an unambiguous manner, namely as the percent of the theoretically possible separation, over a range where the isotope ratio changes from 10:1 to 1:10. This range is symmetrical about a 1:1 isotope ratio and equalizes the weighting of separation characteristics for both isotopes. Using this formulation, a performance number or figure of merit made up of the three part product, Separation $\times$ Efficiency, isotope I $\times$ Efficiency, isotope II, has been proposed.

The chief determinants of performance in a two-channel instrument are the settings of the upper discriminator on the lower channel (UDLC) and the lower discriminator on the upper channel (LDUC) when the gain controls are set to maximize the counting efficiency for each isotope in its characteristic channel. Finding the region of optimum performance and the settings which determined this region required the completion of a matrix of values in which the UDLC setting were the row headings and the LDUC settings are the column headings. Solution of a typical instance, where 15 values for each discriminator were recorded, required 225 performance computations, and a suitable computer program written by M. H. Dipert

has enabled these computations to be performed with ease. The output of values represent an *instrument response surface*, and distinct performance contours are evident in the matrix. Changes in the shape and size of the optimum performance contour as a function of the gain controls and their design, as well as the stability of isotope ratio values in these regions,

are presently under study. It is anticipated that at least three other commercial instruments will soon be available for testing and these measurements will be conducted on them as well. The results of these tests will indicate the present state of the art and enable more meaningful specifications of performance to be defined.

RADIATION SURVIVAL IN MAMMALIAN CELLS IN CULTURE AS A FUNCTION OF AGE IN THE GENERATION CYCLE

Warren K. Sinclair and Richard A. Morton

PURPOSE AND METHODS

Past studies⁽¹⁻⁵⁾ have established that there is a marked variation in the x-ray response of Chinese hamster cells in culture as these cells age between one division and the next. The cells were found to be quite sensitive in G_1 and G_2 and most resistant in the latter part of the S period (Figure 161). Most, but not all, of the variation in response was found to be due to changes in the shoulder rather than in the slope of the survival curve.

The purpose of this investigation is to try to relate changes in x-ray survival to particular states or biochemical processes occurring within the cell at the time and hence, perhaps, to modify such responses.

The method employs a synchronized population of Chinese hamster cells which normally grow in log phase with a generation time of ~ 10 hr ($G_1 = 1.5$ hr, $S = 6$ hr, $G_2 = 1.5$ hr, $M \sim 1$ hr). Immediately after harvesting the synchronized population, cells are at or near zero age and the first cycle continues for a little longer than 10 hr. Responses may be followed for one or more cycles. The criterion of effect is usually the reproductive integrity of single cells scored by the number of colonies formed after a suitable incubation period.

PROGRESS REPORT

Technique Studies

The method used employs both a brief cooling shock (4°C for 1 hour), reincubation, then gentle agitation in 0.03% trypsin. The responses observed have been demonstrated to be independent of these three factors by comparing populations prepared by the usual method with those harvested a) without cooling, b) without trypsin, c) with greater agitation. Varying these factors modifies the yield and the plating efficiency but not the form of the response. The fraction of the population synchronized in this way has also been shown to yield results characteristic of the population as a whole.⁽⁴⁾

The responses have also been shown to be independent of the presence of large numbers of freshly radiation-killed asynchronous cells added at the beginning of the cycle. Furthermore, the x-ray and labeling response

is not affected by the type of medium employed whether EM-15 (Eagles salts and amino acids with fetal calf serum), HUT-15 (EM-15 + NCTC 109 + trypsin), or HUT-15 conditioned by growth of cells in log phase for 24 hours. These factors suggest that cells *probably* move through the cycle at about the same rate (independent of cell concentration).

Relation between Survival and DNA Synthesis Time

Figure 161 shows the results of pulse labeling with tritiated thymidine (3Ci/mM, $0.3 \mu\text{Ci/ml}$) for 15 minutes the population of synchronized cells as a function of time. The response of single cells following 700 R (660 rads) of 250 kvp x-rays is shown on the same time scale. The x-ray survival characteristically trails the passage of the cells into DNA synthesis in both the first and second cycles by 1–3 hours.

Attempts have been made to modify the relationship between x-ray response and uptake of H^3 -thymidine. When the temperature is reduced from 37°C to $\sim 32^\circ\text{C}$, the cycle is expanded from about 12 hours to about 18 hours and the separation between rise in labeling and rise in survival is increased proportionally. When the pH is lowered from 7.3 to 6.8, the cycle is expanded slightly and the x-ray survival occurs slightly later. A more acute lowering of pH may perhaps have shown a separation, but plating efficiency begins to decline below a pH of 6.8. Lowering the content of fetal calf serum from 13% to 5% and to 2% lengthened the cycle from 12 hr to 18 hr and 22 hr, respectively, and shifted the maximum x-ray survival by about the same proportional amount. Consequently, the time relationship between entry of cells into DNA synthesis and x-ray response does not seem to have been modified by these treatments.

Response of G_1 and G_2 Cells and Use of H^3 -Thymidine to Improve Synchrony

It is obvious from Figure 161 that cells synthesizing DNA (i.e., in S) are more resistant than cells in G_1 G_2 . Even a small percentage of cells in S at the time the sensitivity of G_1 and G_2 cells is determined can, there-

FIG. 161.—Response of synchronized Chinese hamster cells throughout two generation cycles after harvesting. $\bigcirc$ — $\bigcirc$, percent of cells pulse labeled with H^3 -thymidine; $\times$ — $\times$, single cell survival after 700 R (660 rads) 250 kvp x-rays.

fore, obscure the true survival of these cells. To improve the degree of synchrony at these times, high specific activity H^3 -thymidine (10–15 Ci/mM) has been added in amounts lethal to any cells synthesizing DNA; 20 μ Ci/ml for 1 hour is sufficient for this purpose. This procedure has enabled the true sensitivity of G_1 cells to be established. In Figure 162, for example, the response of nominally G_1 cells (curve I) is compared with that of G_1 cells only (curve II) from which the S cells were removed by H^3 -thymidine. True G_1 cells evidently are rather more sensitive than previously thought. The shoulder is small (as found previously) and the slope is greater, so that D_0 is reduced to about 160 rads. Preliminary results for true G_2 cells with S cells excluded from the mixed population in the same way indicate that these cells are at least as sensitive as G_1 cells and possibly more so.⁽⁶⁾

Mitotic Cells

The normal technique for obtaining survival data for cells at different ages is not actually suitable for mitotic cells since at the end of the first cycle some measure of asynchrony has already occurred and not enough of the cells are in mitosis at one time to distinguish between them and others. At the beginning of the cycle, the mitotic cells have not yet attached and cannot be examined under the same conditions as attached cells at other stages.

An alternative procedure was adopted in which plates about to be harvested were irradiated at various dose

FIG. 162.—Survival of synchronized cells at about 1 hour after harvesting. Curve I, untreated cells, includes some cells already in S [$n \sim 2.3$, $D_0 \sim 210$ rads]. Curve II, cells treated with H^3 -thymidine in amounts lethal to S cells. Survival curve due to G_1 cells only [$n \sim 2.3$, $D_0 \sim 160$ rads].

levels just prior to harvesting and the harvested population plated. Yield and mitotic index were reasonably constant throughout a series and thus a survival curve

FIG. 163.—Survival of synchronized cells irradiated just prior to harvesting. I, untreated cells, includes some cells actually in *S*; II, cells treated with H^3 -thymidine ($20 \mu\text{Ci/ml}$, 15.3 Ci/mmole) for 1 hour prior to irradiation and harvesting.

for mitotic cells could be established. Again, the presence of a few *S* cells, because of their relatively high resistance, has proved to be a major source of error. To correct for these cells, H^3 -thymidine in lethal amounts was added to the plates 1 hour before exposure (and then harvesting) in two instances as shown in Figure 163. If these preliminary data are confirmed, mitotic cells will be found to be most sensitive of all, with little or no shoulder and a slope corresponding to a D_0 of ~ 140 rads.

Responses in Other Cell Lines

Terasima and Tolmach⁽⁷⁾ and Whitmore *et al.*⁽⁸⁾ have described the x-ray response for HeLa cells and *L* cells, respectively. Although some features of the response differ with those reported by us for Chinese hamster cells, there are common features. The most notable is the fact that in all three cell lines survival rises soon after DNA synthesis begins and, in fact, the response during *S*, G_2 , and *M* is broadly, at least, rather similar in the three lines. In HeLa cells and to a less extent in *L* cells, however, a period of x-ray resistance is evident during G_1 . This might be the case in Chinese hamster cells, too, if their G_1 were long enough. To test this possibility a "small-colony" subline of Chinese hamster cells⁽⁹⁾ with a naturally longer G_1 [$G_1 = 6\frac{1}{2}$ hr, $S \sim 12$ hr, $G_2 = 2\frac{1}{2}$ hr, $M \sim 1$ hr] was synchronized and the x-ray response examined (Figure 164). This line was found to have a period of G_1 resistance similar to that observed in HeLa cells and *L* cells. The usual resist-

FIG. 164.—X-ray response with time after harvesting synchronized population of Chinese hamster cells, small colony subline with longer than normal generation time. (Dose, 520 rads.)

ant period in S was also found and some further fine structure in the survival response between S and G_2 . This at least serves to establish that the existence of a resistant phase during G_1 is not species dependent but probably depends on the length of G_1 .

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SURVIVAL OF CHINESE HAMSTER CELLS AFTER EXPOSURE TO MONOENERGETIC NEUTRONS

Warren K. Sinclair and Norman A. Frigerio

PURPOSE AND METHODS

Mammalian cell survival curves characteristically possess a pronounced shoulder followed by an exponential region when low LET radiation is used. After high LET radiation, the survival curve usually has no shoulder and survival declines exponentially with dose. Nevertheless, such a curve can be the result of two or more multihit components, especially if the resolution of the data is poor close to the origin. True single hit kinetics at high LET imply no resolution into components, and, therefore, no variation in response throughout the generation cycle is possible. Furthermore, if there are no components with a shoulder indicative of a sublethal damage process, no repair is possible and two-dose exposures, with intervals between fractions, should yield the same survival as the single total dose. In order to test these predictions, a synchronized population of cells is greatly superior to asynchronous populations, since it enables the response throughout the generation cycle to be determined unequivocally and the two-dose responses to be established free of complications occasioned by the effect of cell progression through the cycle.

The source of high LET radiation was neutrons of about 555 keV (± 100 keV) produced by protons in a Van de Graaff of ~ 2.38 MeV striking a lithium target [$Li^7(p,n)Be^7$].⁽¹⁾ The physical conditions of exposure are such that only one plate (52-mm plastic) containing

cells can be exposed at one time and the beam is scanned over the surface of the plate which must be mounted vertically. The maximum dose rate with the equipment available was 10-12 rads/minute. The medium must be removed from the plate to carry out the exposure, and in long exposures moisture is lost more quickly from the top of the plate than the bottom and cells die. Rotation of the plate, even slowly, avoids this.

Dosimetry was carried out using a fission counter of Uranium-235⁽²⁾ supported by measurements with a calibrated tissue-equivalent chamber. Exposures were controlled by the total charge collected from the proton beam in a Faraday cup calibrated by the fission counter.

Neutrons of 555 keV (± 100 keV) deliver most of their dose at an LET between 40 keV/ μ and 90 keV/ μ with a dose average LET value of about 70 keV/ μ .⁽³⁾ This value is close to that at which cells are believed to have maximum sensitivity.

PROGRESS REPORT

Several runs have been made aiming at survival curves for asynchronous cells. These have been limited by the physical conditions of exposure, the performance of the Van de Graaff itself, and its very limited availability. The nonavailability of a suitable area in the vicinity of the machine for emptying and filling plates with medium under sterile conditions has also severely limited accuracy. Nevertheless, preliminary survival

FIG. 165.—Survival of asynchronous Chinese hamster cells after exposure to monoenergetic neutrons of 555 keV. The points for different experiments vary in quality, those for Exp. 273, marked X, being considered the most reliable. The curve seems to be a simple exponential with a reciprocal slope of about 67 rads.

data on asynchronous cells are displayed in Figure 165, some of the points being more reliable than others. The survival curve appears reasonably close to a simple exponential with a reciprocal slope corresponding to about 67 rads. More complete experiments are needed before these preliminary data can be considered established however.

With synchronized cells, timing and availability of the Van de Graaff are most important. One run only (a

rather poor one because of failure of the Van de Graaff at the critical starting time) has been conducted, and the results cannot be considered sufficiently reliable to report.

The survival of Chinese hamster cells after exposure to x-rays and to neutrons for the asynchronous population evidently yields an RBE of ~ 3 (200 rads/67 rads) when slopes only are compared. This is a common but erroneous practice with RBE determinations. If the shoulder also is taken into account, the RBE depends on the level chosen for comparison. It can be shown⁽⁴⁾ that, if the survival curves fit single-hit multitarget expressions

$$\text{RBE} = \frac{D_r}{D_t} = \frac{(D_0)_r}{(D_0)_t} \left[1 + \frac{\log_e \frac{n_t}{n_r}}{\frac{D_t}{(D_0)_t}} \right]$$

where r and t refer to reference and test radiation, respectively; n is the extrapolation number and D_0 the reciprocal of the slope; e.g., for a neutron dose of 100 rads, the expression in the bracket reduces to 1.90 and the RBE becomes 5.7. For smaller neutron doses, the RBE is higher still, but as the neutron dose is raised the RBE falls to a value approaching 3.

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REPAIR OF X-RAY DAMAGE IN SYNCHRONIZED MAMMALIAN CELLS IN CULTURE

Warren K. Sinclair and Richard A. Morton

PURPOSE AND METHODS

In the previous Annual Report⁽¹⁾ studies were described using synchronized Chinese hamster cells in which it was established that cells in S can repair sublethal x-ray damage rapidly and completely within a short time (i.e., by the next division) after exposure. Furthermore, repair examined by the two-dose tech-

nique and found to occur at all stages of the cycle could be synthesized to yield the expected response in an asynchronous population. This was shown to agree well with experiment.

It was, however, made clear that while cells appear to recover at all stages of the cycle, this could not be considered proved because the effect of a few resistant

S cells could obscure the true behavior of G_1 and G_2 cells. It is important, with regard to the mechanism of the repair process, to establish whether, for example, cells not synthesizing DNA (e.g., G_1 cells) can repair sublethal damage and if so, can they do so while still in G_1 or is a period of DNA synthesis necessary?

PROGRESS REPORT

In order to examine the true two-dose x-ray response of G_1 and G_2 cells, H^3 -thymidine in amounts lethal to S cells ($20 \mu\text{Ci/ml}$, $10\text{--}15 \text{ Ci/mM}$ for 1 hour) was added. Plates treated in this fashion were exposed to 1000 R and to $500 \text{ R} + \tau + 500 \text{ R}$ with various intervals, t , be-

tween the two doses. Control plates received H^3 -thymidine only. To date, such experiments have been carried out in both G_1 and G_2 , but the difficulty of estimating the expected survivals after such complex treatments has so far been such that only preliminary results have been obtained. More complete experiments are expected to be carried out in the near future.

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SIZE CHANGES IN MAMMALIAN CELLS DURING THE GENERATION CYCLE

Warren K. Sinclair and Patricia Coleman

PURPOSE AND METHODS

As a cell grows between one division and the next, the cell contents must double, and the cell just before division must have twice the mass and twice the volume of daughter cells just after division. Between these two extremes, the cell may grow in volume or mass uniformly or nonuniformly. Previous work on mammalian cells (HeLa cells) by Sandritter *et al.*,⁽¹⁾ who observed single cells by interference microscopy, showed that both mass and volume increased rapidly just after

mitosis to about three times the initial value about $\frac{2}{3}$ of the way through interphase. Thereafter, mass and volume declined to about twice the initial value just prior to mitosis. The changes observed are believed to be mainly due to water intake of the cell. A similar result was found for HeLa cells by Terasima and Tolmach.⁽²⁾

The variation of cell size under various stresses is often indicative of damage and is, therefore, a useful parameter to be able to measure, e.g., see the cell size

FIG. 166.—Relative cell volume vs. relative cell number of partially synchronized hamster cell population as a function of time. Panels (a)–(k), $\sim 11,000$ cells/ml; (l) 12,500, (m) 14,200; (n) 16,200, (o) 17,200. (Coulter counter set at Amp 1; Ap.C. 8; Scale 6; 4 seconds.)

FIG. 167.—Peak channel number on Coulter size plotter (proportional to cell volume) against time for partially synchronized hamster cells. In this experiment not all the harvested cells had separated by 1 hour after harvesting. The increase covers a factor of almost two in volume. Note: Channel 8 on the plotter corresponds to a cell of about 11.7μ in diameter.

changes for *S* cells killed by hydroxyurea.⁽³⁾ For this reason, the examination of cell size changes in a synchronized population with time (and perhaps later under stresses such as drugs and radiation) was considered valuable.

The method differs from the observation of single cells microscopically by Sandritter *et al.*,⁽¹⁾ in which the cells are not disturbed. In this instance, cells synchronized by the usual method are inoculated onto plates and then reincubated. At intervals thereafter, plates are removed from the incubator, trypsinized and pipetted to form a suspension. The concentration of cells and the size distribution of the population was then found by sizing on a Coulter counter distribution plotter (which uses 25 single channels in sequence) and on a Coulter counter run into an analyzer which records in 100 channels simultaneously. The two methods yielded very similar results, the resolution of these two sets of apparatus being about the same [unfortunately inferior to much more elaborate equipment now available], but the multichannel analyzer permitted a much smaller sample to be used.

PROGRESS REPORT

Preliminary experiments only have been possible with the present apparatus. The results recorded on the Coulter counter size distribution plotter for one experi-

ment are shown in Figure 166. A steady increase in the average volume of the cell population occurs throughout the generation cycle. This is indicated also by the plot of peak channel number versus time (Figure 167). There is a sharp drop in volume as the cells go through mitosis at about 10 hours but prior to that time the rate of volume change was essentially constant. The cells are observed in a trypsinized suspension, and their normal condition is, of course, modified to one in which conditions are constant throughout the cycle. Consequently, any effects of changing water content, etc., may not be observed by this technique. The volume and mass are likely to bear a constant relationship.

This experiment needs to be repeated under better synchrony conditions and preferably with apparatus of higher resolution before the method is applied to the examination of stresses.

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HYDROXYUREA: DIFFERENTIAL LETHAL EFFECTS ON CULTURED MAMMALIAN CELLS DURING THE CELL CYCLE

Warren K. Sinclair

PURPOSE AND METHODS

Hydroxyurea, a drug under test as a cancer chemotherapeutic agent, has recently been shown to inhibit incorporation of thymidine into the DNA of mammalian cells⁽¹⁾ and then to block DNA synthesis even in the presence of moderate amounts of thymidine. The drug is, therefore, of interest in the study of x-radiation effects in synchronized hamster cells because DNA-blocking agents may provide clues to the radiation resistance of hamster cells during the latter part of the S period. Such an agent may also shed some light on the process of repair of sublethal damage in mammalian cells.

Initial studies with hydroxyurea in asynchronous and then in synchronized populations established that not only did the drug effectively block the uptake of H³-thymidine in hamster cells about to go into DNA synthesis, but it had a lethal effect on cells already in synthesis. Although this discovery⁽²⁾ rendered the drug less useful for its intended purpose, it also opened up a new area of investigation and potential application for this agent.

PROGRESS REPORT

Inhibitory and Lethal Effect of Hydroxyurea

Initial studies in asynchronous populations of Chinese hamster cells in culture using concentrations of

10^{-3} , 10^{-4} , and 10^{-5} M hydroxyurea showed that while 10^{-5} M was ineffective and 10^{-4} M only partially effective, 10^{-3} M completely inhibited DNA synthesis (in less than 1/2 hour). 10^{-3} M also caused a 60% reduction in cell survival, but equally after an exposure of either 3 hours or 6 hours. These facts suggested that among the cells exposed to the drug, S cells alone failed to survive and that other cells were blocked and prevented from entering S, but their survival was not impaired.

Studies using synchronized populations then showed clearly (Figure 168) that at 5×10^{-4} M or 10^{-3} M for 1 hour S cells are effectively destroyed; G₁ cells are blocked from entering S and G₂ and M mitotic cells move until the end of G₁ where they too are blocked from entering S. Details of the effects of hydroxyurea on S cells with time and with drug concentration are available in the reference cited.⁽²⁾

The inhibitory effect of hydroxyurea is shown in Figure 169. During 5 hours of exposure to the drug, no cells take up a pulse label of H³-thymidine but as soon as hydroxyurea is removed, the number of cells synthesizing DNA rises rapidly to 100%. (The 15% or so of cells synthesizing DNA at 1.2 hours when hydroxyurea was added are destined not to survive, but they nevertheless take up thymidine again as soon as the drug is removed.) Evidently the degree of synchrony in the population is quite markedly improved by the ap-

FIG. 168.—Survival of G₁, S, and G₂, Chinese hamster cells in culture after different lengths of exposure to hydroxyurea. Survival is relative to the survival of untreated cells, plating efficiency ~50%.

FIG. 169.—Percent of synchronized Chinese hamster cells pulse labeled with tritiated thymidine (15 minutes, $0.3 \mu\text{Ci/ml}$, 3 Ci/mM) with time. Curve A, no hydroxyurea treatment; Curve B, hydroxyurea, $5 \times 10^{-4} \text{ M}$ for 5 hours starting at 1.2 hours (G_1 cells).

plication of hydroxyurea in G_1 , the labeling index, L , of curve A, $\sim 55\%$ improving to $\sim 83\%$ for L_H of curve B.

Hydroxyurea as a Synchronizing Agent

The results above suggest that hydroxyurea is likely to be a most effective synchronizing agent in asynchronous populations since the proliferative capacity of S cells can be destroyed while the remaining cells ($\sim 40\%$ in hamster cell populations, but much higher in cell lines with long generation times) merely pile up in G_1 . Thus it is reasonable to expect better synchrony than is obtainable with inhibitors such as FUDR or amethopterin⁽³⁾ which block S cells but may permit them to survive. The use of hydroxyurea has an advantage over methods involving suicide with lethal amounts of H^3 -thymidine because in the latter there

is no inhibitory action resulting in accumulation of surviving cells. Any inhibitory drug such as hydroxyurea has the inherent disadvantage, however, that properties of surviving cells may be modified and their response to other agents (e.g., x-rays) may not be the same as those of the untreated population.

Preliminary experiments indicate that the predicted synchronizing properties of hydroxyurea will be borne out. In Figure 170 are shown the results of one experiment in which asynchronous cells were exposed to hydroxyurea for 6 hours. Cells were plated for growth of survivors and pulse labeled with H^3 -thymidine. Marked synchrony is apparent in the growth curve of the treated cells; furthermore, the percentage uptake of H^3 -thymidine (which includes S cells that are destined to die; will, therefore, not reach 0% after the treatment) falls to its minimum value at about the same time as the cells

FIG. 170.—Hydroxyurea ($10^{-3} M$) as a synchronizing agent in an asynchronous population of Chinese hamster cells. $\circ$ — $\circ$ Surviving colonies from cells plated from untreated dishes. $\times$ — $\times$ Surviving colonies from cells plated from dishes treated with $10^{-3} M$ hydroxyurea for 6 hours. $\square$ — $\square$ Percentage of cells labeled with H^3 -thymidine on dishes treated with hydroxyurea ($10^{-3} M$) for 6 hours.

first divide. Furthermore, the first division after the drug is removed (i.e. ~ 7 hours later) is not delayed at all for cells leaving G_1 , and thus the growth properties of the survivors appear unaffected.

The Fate of S Cells Exposed to Hydroxyurea

Evidently cells in S exposed to $10^{-3} M$ hydroxyurea for 1 hour are unable to proliferate. Do they pass through one or more divisions; do they die at once or perhaps persist for a considerable period without dividing? To answer this question, synchronized hamster cells were exposed during S for $2\frac{1}{2}$ hours (6–8.5 hours after harvesting) to $10^{-3} M$ hydroxyurea and size changes and the growth of cells subsequently followed in time. The results are shown in Figure 171 in which successive panels show the change in size and number of the cell population. At 6 hours this population was not perfectly synchronized, about 85% of the cells being in S; the 15% of the cells not in S will not be killed

by the drug. As can be seen, there is no change in cell number between 8.5 hours (panel c) and 16 hours (panel e) but the average size of the cells increases. By 22 hours, the cell number has increased and a new group of smaller cells has appeared. The two groups evident in panel (f) are first, the smaller viable cells which were not in S during the period of the administration of the drug; and second, the enlarging but nondividing S cells which are destined to die. As time goes on, the small cell size group keeps on increasing in number (and varying somewhat in size because they are synchronized) while the large group keeps expanding in size and from ~ 30 hours onwards decreasing in number. At this same time (30 hours post-zero), particulate matter keeps increasing in the medium above the cells. This matter represents dead cells and parts of cells originally in S at 6 hours. By 46 hours practically none of the large cells is left in the population. Thus it appears that S cells exposed to hydroxyurea die *without dividing* about 30–40 hours after the treatment is applied.

FIG. 171.—Changes in cell population volume for synchronized Chinese hamster cells exposed to hydroxyurea, $10^{-3} M$, for 2½ hours (from age 6 hours–8.5 hours after harvest). Coulter size distribution plotter, Amp 2; Ap.C. 8; LT 12; UT 50; Scale 6.

Application of Differential Lethality to *in vivo* Use of Hydroxyurea

The lethal effect of hydroxyurea on *S* cells *in vitro* presumably has an important bearing on the behavior of the drug *in vivo*, particularly with regard to its anti-tumor action and the complications of treating humans. Treatment of human cancer patients with hydroxyurea involves doses within the range 40–100 mg/kg/day. If the whole of the daily dose were distributed uniformly in all tissues (at least temporarily) before elimination or detoxification of the drug, a concentration of about $5 \times 10^{-4} M$ to $1.3 \times 10^{-3} M$ would result. If the data presented here are applicable, these concentrations should be quite sufficient to cause death of cells in *S*, inhibition of cells in *G*₁ and in time, accumulation of other cells in *G*₁. Thus, if this concentration of hydroxyurea actually reaches proliferating tumor cells and is maintained there for some hours (information is needed on this point), significant damage should, and apparently

in some instances does, result.⁽⁴⁾ Other nontumor proliferating cells in the body (such as bone marrow cells and some cells of the gastrointestinal tract) should suffer the same fate. Therefore, it is not surprising that clinically, depression of bone marrow activity and distressing gastrointestinal symptoms have often been found. These complications tend to limit patient therapy with hydroxyurea.⁽⁴⁾

Continued exposure to hydroxyurea at these levels must eventually be fatal even to inhibited *G*₁ cells: for example, I have some preliminary evidence in Chinese hamster cells *in vitro* that *G*₁ cells do not survive 24-hour treatment with $10^{-3} M$ hydroxyurea. It is interesting, nevertheless, to speculate that tumor treatment with hydroxyurea may be much more effective if another agent, preferentially lethal to cells at stages other than *S* could be used in conjunction with it, especially if this agent can be confined primarily to the tumor cells or region. For aerobic cells at least, x-rays

would seem to be a good choice since not only in Chinese hamster cells⁽⁵⁾ but also in HeLa cells⁽⁶⁾ the latter part of the *S* period is the most resistant to x-rays and cells in late *G*₁ just before entering *S* are quite sensitive. An effective treatment technique may be, therefore, to administer hydroxyurea and follow this, at a time roughly equal to the generation time minus the length of the *S* period, with x-radiation. This x-radiation should preferably be delivered locally to tumor cells to avoid increasing the complications in bone marrow and intestinal cells.

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SURVIVAL OF X-IRRADIATED CHINESE HAMSTER CELLS IN VITRO AT LOW LEVELS BY CLONING TECHNIQUE

Warren K. Sinclair and Grace Racster

PURPOSE AND METHODS

Quantitative *in vitro* mammalian cell culture techniques, in which survival following a treatment such as radiation exposure is scored by the observation of colonies formed after a given incubation period, become more difficult at low survival levels. In radiation studies *in vitro* difficulties are usually encountered at survival levels lower than about 10^{-3} or 10^{-4} . Since the shape of survival curves may be quite seriously affected by incorrect (usually lower) survivals at high doses, it is necessary to examine various technical factors at such low levels which may have been unimportant at higher survival levels. These include 1) the effects of the damage giving rise to small colony formation,⁽¹⁾ which occurs in almost 100% of the cells at survival levels of the order of 10^{-5} and lower, 2) possible hypoxia of the many cells needed, and 3) the depletion of the medium due to its use by cells destined to die. In an environment in which dying cells are depleting the medium, potential surviving cells may not be able to survive, whereas fresh medium at the appropriate time may encourage survival. The latter point has been noted by Berry in connection with HeLa cells.⁽²⁾

In Chinese hamster cells, if the survival curve continues to be exponential below $\sim 10^{-3}$,⁽¹⁾ it is possible to predict the survivals at about 2500 R (10^{-5}) and 3000 R (10^{-6}). The method is to investigate survivals at these dose levels by varying the length of incubation, the medium conditions in the dish and other technical factors in order to determine true survivals free of artefacts.

PROGRESS REPORT

With normal survival and incubation techniques, very few survivors have been scored after 2500 R and none at all after 3000 R. However, after 2500 R, increasing the length of the incubation period from about 10-12 days to 4 weeks increased survival by a factor of 3 on undisturbed plates. We have shown this to be due to depletion of the medium, which supports the growth of unirradiated cells poorly.

After 3000 R (2840 rads), we expect the survival to be about 10^{-6} . Survivors have been obtained only by changing the depleted medium to fresh medium. Such a change is effective only after about 6-7 days, but continues effective up to perhaps 20 days, allowing a further 10-14 days in fresh medium for the development of colonies. The constancy of response during these times indicates that the colonies scored are not the result of reseeding. The survivals found after 3000 R are of the order of 10^{-6} .⁽³⁾ The investigation is proceeding in order to establish accurate and reproducible survival levels at doses in the 2500 R-3000 R range.

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CYTOLOGICAL STUDIES WITH IONIZING RADIATION
IN MAMMALIAN CELLS IN VITRO I.
CHROMOSOMES OF X-RAY-INDUCED GIANT CELLS

C. K. Yu

PURPOSE AND METHODS

The lethal process induced by x-irradiation in cells *in vitro* includes affected cells which continue to grow to a maximum size and then die (giant cells). Although some descriptions of giant cells have been made, they are not yet well defined. Experiments were, therefore,

performed to study the nature of these cells and especially their chromosome status.

Two established cell sublines, 122D₁ (near diploid) and 122T (near tetraploid), of the Chinese hamster (*Cricetulus griseus*) with single karyotypes were used for this study.⁽¹⁾ Cells cultured at log-phase growth

FIG. 172.—Cells varying in size after x-irradiation

were exposed to 250 kvp x-rays (half value layer, 0.9 mm Cu) at 0, 250, 500, 1000, or 1500 R with a dose rate of about 110 R/minute after which they were sampled for cytological examination. Cytological preparation involved colchicinization, hypotonic treatment, acetic alcohol fixation, drying in air, and staining with acetic orcein.

PROGRESS REPORT

The giant cells induced in both the near-diploid and near-tetraploid cell sublines show no essential cytological differences. The giant cells observed during interphase vary considerably in cell size (Figure 172). At mitosis, they are polyploid cells with up to more than 700 chromosomes (64N?).⁽²⁾ Two types of polyploid cells are observed: the true polyploid type (its chromosomes have already doubled or multiplied); and the endoreduplication type (with doubled or further-multiplied individual chromosomes still side-by-side as occurs in the diplochromosome configuration).⁽³⁾ Both types have massive chromosome aberrations (chromatid or chromosome deletions, etc.). Polycentric chromosomes occur frequently in the giant cells, especially in those with a highly polyploid form. There are two types of polycentric chromosomes: the chromatid type (a complex configuration of chromatid interchanges that results from multiple chromatid breakages, but with many chromosomes involved) and the chromosome type (a number of interstitial centromeric segments that result from chromosome breaks that have joined together). The chromosome type of polycentric chromosomes may be further subdivided into two forms: the simple chain form (i.e., a number of centromeric segments joined together at their broken ends) and the bilaterally identically symmetric (BIS) polycentric chromosomes (their two halves are mirror images of each other).⁽⁴⁾ Both types of polycentric chromosomes are obtained between 26 and 74 hours after exposure to doses of more than 1000 R. They decrease in number by 146 hours after the exposure. The absolute frequency of polycentric chromosomes at a given dose and scoring time appears not to be a reliable criterion because some may break down into mono- or dicentric chromosomes. The relative frequency of this "breakdown" is not known. Of special interest, however, is the mechanism that leads to the formation of polycentric chromosomes.

ism that leads to the formation of polycentric chromosomes.

The formation of chromosome-type polycentric chromosomes may be the result of numerous breaks in chromosomes followed by union of the broken segments. Polycentric chromosomes may be induced in cells which are exposed to x-rays at early interphase, e.g., G_1 before chromatids are formed. If these damaged cells do not die immediately after the exposure, they may enter the S period once, twice, or even more times without a cell division. As a result, the polycentric chromosomes appear to replicate themselves. The replicated polycentric chromosomes also can join together at a broken end to form BIS polycentric chromosomes. If the S periods repeat without division, BIS polycentric chromosomes may be doubled or multiplied depending upon the number of S periods repeated. This repetition process also contributes to a continuous growth and increase in both the size of the nucleus and the cell. An increase in cell size depends upon a repetition of endomitosis. The frequency of the process is radiation dose dependent and in some severely damaged cells, and especially in those cells with BIS polycentric chromosomes many incidents of endomitoses have occurred. Giant cells exist only temporarily in an x-irradiated population and eventually die. Thus they represent one way in which a cell may reach a terminal phase following radiation exposure. Not all cells destined to die as a result of irradiation suffer this fate, however. Other forms of damage and modes of death appear to be possible.⁽⁵⁾

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CYTOLOGICAL STUDIES WITH IONIZING RADIATION IN MAMMALIAN CELLS IN VITRO. II. CHANGES INDUCED BY X-RAYS AT VARIOUS STAGES OF THE CELL CYCLE

C. K. Yu and Warren K. Sinclair

PURPOSE AND METHODS

Synchronization in mammalian cells in culture offers unique advantages for the study of physiological, biochemical, and cytogenetic events occurring during the cell cycle. Experiments are, therefore, designed to establish the cytogenetic changes induced by x-irradiation when synchronized cells are exposed to x-radiation at various stages of the cell cycle.

The V79-285B cell strain of Chinese hamster with a single karyotype of 23 chromosomes is being used for this study. The technique of synchronization has been described elsewhere⁽¹⁾ and provides cells at or near mitosis which can then be followed through subsequent generation cycles. The synchronized cultures are irradiated at different times after harvesting, i.e., as cells proceed into G_1 , early S , late S , and G_2 . They are exposed to 500 R with 250 kvp x-rays (half-value layer

0.9 mm Cu) at a dose rate of 110 R/minute. After x-irradiation, cells are reincubated. Cytological examination is performed when the cells have moved on to mitosis. Initially it has been necessary to examine cultures over a range of times in order to establish the optimum mitotic index.

PROGRESS REPORT

The preliminary results have shown that the division delay caused by x-irradiation is different when the cells are exposed to x-rays at different stages of the cell cycle. Cells exposed to x-rays during G_1 have a shorter division delay than cells x-rayed at later stages of the cycle. Approximately one hour's division delay is observed in the cell population which was x-irradiated during G_1 (one hour after incubating the synchronized population). Cells x-rayed during early S (four hours after incubation) show about two hours' division delay (Figure 173). Cells x-rayed during late S (seven hours after synchronization) seem to have a much longer division delay. A comprehensive study of this sort of radiation effect is currently being conducted. In some cases, the percentage of mitoses is higher in x-irradiated populations than in nonirradiated populations, possibly because the mitotic period has been lengthened by the x-irradiation. The chromosome type of aberration is most frequently obtained when the cells are x-irradiated during G_1 , and the chromatid type predominates when cells are x-irradiated during early S . Further studies of the relationship between chromosome aberrations and cells irradiated at various stages of the generation cycle are in progress.

FIG. 173.—Mitotic indices of a synchronized population sampled at various times after reincubation. The mitotic peaks of cells irradiated during G_1 (circles and broken line) and during early S (crosses and dotted line) occur at the 12th and 13th hour, respectively; that of control seems to occur at the 11th hour (dots and straight line).

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THE CHEMICAL SHIFTS AND CHARGE DENSITIES IN NITROGEN HETEROCYCLES

Steven S. Danyluk and David J. Blears

PURPOSE AND METHODS

Phenazine(I) *o*-phenanthroline(II), quinoxaline(III), and 2-3-diphenylquinoxaline(IV) are members of a series of nitrogen heterocycles whose spectroscopic properties, i.e., fluorescence, and phosphorescence, are strongly dependent upon solvent, concentration, and pH.^(1, 2, 3) In common with other nitrogen heterocycles the above compounds also show a marked activity in many biological systems.⁽⁴⁾

It was suggested,⁽⁵⁾ on the basis of theoretical considerations, that the unusual properties of these compounds are due to the presence of lone-pair electrons on the nitrogen and their effect upon the π electron distribution in the ring system.

However, reliable data for π electron densities in N heterocyclic compounds is lacking and the objective of the present investigation was to determine the electron distribution in a selected series of such compounds

FIG. 174.—Proton magnetic resonance spectrum for quinoxaline in CH_2Cl_2 ; concentration, 5 M%. Upper curve, observed spectrum; lower curve, calculated spectrum.

FIG. 175.—Proton magnetic resonance spectrum for the quinoxaline cation in trifluoroacetic acid. Upper curve, observed spectrum; lower curve, calculated spectrum.

and to compare the results with values predicted by molecular orbital (MO) calculations.

PROGRESS REPORT

In earlier work^(6, 7) it was shown that under certain conditions the chemical shifts in aromatic hydrocarbons and in pyridine⁽⁸⁾ are directly related to the π electron densities at the adjacent carbon atom. A similar correlation would be expected to hold for the polycyclic N heterocycles, providing factors such as magnetic anisotropy and electric field effects due to the N atom are taken into account.⁽⁸⁾ Accordingly, the proton high-resolution spectra were measured for compounds I to IV at low concentrations in an inert solvent (CH_2Cl_2) at 36°C. The spectra were also recorded in a strongly protonating solvent, CF_3COOH over the temperature range -27° to 36°C. The spectra were analyzed by an iterative procedure⁽⁹⁾ with the aid of the CDC 3600 computer to yield chemical shifts and coupling constants accurate to ± 0.5 and ± 0.05 cps, respectively. An example of the fit obtained between observed and calculated spectra for quinoxaline in CH_2Cl_2 is shown in Figure 174.

The chemical shifts for the heterocycles studied in this work generally lie in the range observed for pyridine derivatives.⁽⁸⁾ The chemical shifts in II increase in the same order as in pyridine,⁽⁸⁾ i.e., $\beta > \gamma > \alpha$, while the chemical shifts in compounds I, III, and IV are in the order $\gamma > \beta$. The ortho, meta, and para coupling constants noted for compounds I-IV are similar to those observed in pyridine.⁽⁸⁾ In each case protonation of the heterocycle occurs at the N atom and leads to a significant shift to low field of all of the protons. This low-field shift is accompanied by a decrease in relative shift between the ring protons as is evident in the spectrum for protonated quinoxaline shown in Figure 175.

CONFIGURATIONAL CHANGES OF BIOPOLYMERS. I. HEAT-INDUCED CHANGES IN GLOBULAR PROTEINS*

Steven S. Danyluk and David J. Blears

PURPOSE AND METHODS

Many globular proteins are unstable to excessive heat, and such treatment generally causes a transition of the protein into a more disoriented state in aqueous solution.⁽¹⁻³⁾ In this transition the segmental mobility of the disordered regions increases and a greater number of polar and apolar groups are exposed to the solvent. Both of the changes will affect the local structure of the water in the hydration sheath surrounding the

CONCLUSIONS

The chemical shifts for the protons in the free bases are found to be appreciably influenced by 1) the magnetic anisotropy of the N atom, 2) the electric field of the N atom ($\text{N}-\text{H}^+$ bond in protonated bases), and 3) the ring current of adjacent rings. When corrections are made for each of these factors, a good correlation is obtained between the chemical shifts and calculated electron densities. In the protonated species the decrease in relative shifts indicates that the π electron density is more uniformly distributed around the ring in agreement with results obtained for aromatic hydrocarbons.⁽⁷⁾

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protein. In the former case, the increased segmental mobility causes a disruption in the "icelike" nature of the surrounding water, while in the latter case, exposure of additional groups increases the number of water molecules bound in the hydration layer. If the extent of disordering in the protein is sufficiently large, the changes induced in the solvent can be expected to affect the nuclear spin-lattice relaxation rate of the water molecules. In principle, therefore, it should be possible to follow structural changes in the protein by measuring the proton spin-lattice relaxation time of the water

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FIG. 176.—Variation of T_1 of the water protons in aqueous solutions of $5\times$ RNase: (A) 14 mg/ml sample; (B) 16 mg/ml sample; (C) 21 mg/ml sample. The time spent at each temperature before cooling to 22°C is shown in hours at each point. The temperature axis represents the temperature at which the heating cycle was performed.

molecules; the purpose of this study was to test this hypothesis for a number of globular proteins.

The spin-lattice relaxation times for water protons in aqueous solutions (1–10%) of $5\times$ RNase (recrystallized five times) monomeric RNase, and human serum albumin (HSA) were measured by the spin-echo technique using a Varian DP60 high-resolution spectrometer modified for pulse studies.⁽⁴⁾ The solutions were subjected to periods of controlled heating at selected temperatures in the range 22 – 100°C , cooled at 22°C , and the spin-lattice relaxation times were measured at 22°C . The samples were generally heated at the selected temperature for 15–30 minutes, and the elapsed time in the cooling step, prior to measurement, ranged from 5 to 10 minutes. The effect of heat treatment of RNase solutions was also followed by recording the UV spectra for these solutions.

PROGRESS REPORT

The spin-lattice relaxation times, T_1 , for both the RNase and HSA solutions were found to change in a manner characteristic of the protein. This is illustrated in Figures 176 and 177 where the T_1 changes on heat treatment are shown for various concentrations of $5\times$ RNase and HSA, respectively. In both cases the relaxation time-temperature curves are sigmoid in appearance

with a rapid increase in T_1 occurring over a relatively narrow temperature range ~ 15 – 20°C . The increase in T_1 for RNase solutions heated above 40°C is accompanied by a shift in the absorption maximum for the

tyrosyl group from $277.5\text{ m}\mu$ to $276.3\text{ m}\mu$. The T_1 's of RNase solutions heated above 40°C gradually decrease with time and approach the original value at 22°C .

The increase of T_1 with heat treatment can be interpreted on the basis of a decrease in the correlation time, T_c , for the fluctuating dipolar fields at the water protons.⁽⁵⁾ In the RNase and HSA solutions this decrease is due to the changes induced in the solvation layer by the increased inter- and intra-molecular motion of the protein. Both the increased rotation rate of the macromolecule in solution and the high degree of internal flexibility acquired by the segments and/or side chain act to disrupt the local structure of the water in the regions surrounding the protein and thus decrease T_c . The increase in internal mobility is confirmed by the marked narrowing in line widths noted in the continuous-wave high-resolution spectra for the heat-treated proteins. From the latter the upper limit for segment reorientations is estimated to be considerably less than 10^{-4} seconds.

Although the shift of the tyrosyl peak in RNase solution to shorter wavelengths strongly suggests a denaturation process similar to that observed in urea solutions,⁽⁶⁾ the detailed mechanism cannot be established unequivocally. The fact that T_1 is stabilized at higher values after heating and then decreases over a finite period of time to the original value at 22°C indicates that other complicating effects are involved. The most likely possibilities are aggregation-disaggregation equilibria⁽⁷⁾ and disulfide bond rearrangements.⁽⁸⁾ Of these

FIG. 177.—Variation of T_1 of the water protons in aqueous solutions of HSA: (A) 120 mg/ml sample; (B) 24 mg/ml sample. The time spent at each temperature before cooling to 22°C is shown in hours at each point. The temperature axis represents the temperature at which the heating was performed.

the aggregation process would be more favored and would account for the difference between initial T_1 values for monomer and 5×RNase solutions.

One additional point of interest is the surprisingly narrow temperature range, ~15°C, in which the T_1 transition occurs. Similar relatively abrupt changes with temperature have been noted in the line width of high-resolution spectra for synthetic polynucleotides and transfer RNA⁽⁹⁾ and have been interpreted in terms of a highly cooperative transition between ordered and disordered state of the polynucleotide. It seems likely that the disordering of the proteins upon heating occurs in an analogous manner.

CONCLUSIONS

The results of this work have shown the feasibility of studying organizational changes in proteins by measuring the changes in spin-lattice relaxation times of solvent molecules using pulsed nuclear magnetic resonance (NMR) techniques. Since the time scale of molecular events which can be studied effectively by pulsed NMR covers a very wide range $>10^6$ seconds, the information content of T_1 measurements is inherently much greater than that of other physicochemical techniques. In future work this advantage will be

exploited to study configurational changes in other proteins, nucleic acids, and RNA-protein complexes.

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THE STRUCTURE AND STABILITY OF MOLECULAR COMPLEXES FORMED BY NUCLEIC ACIDS AND PROTEINS. I. THE BINDING OF ACRIDINE TO DNA

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PURPOSE AND METHODS

The mutagenic^(1, 2, 3) and carcinogenic^(4, 5) effects of acridines on biological systems is well documented. In order to account for the particular effectiveness of the acridines as mutagens Lerman has proposed that these compounds bind to DNA by intercalation between adjacent nucleotide-pair layers in the helix.^(1, 6) This hypothesis was supported by evidence which showed that the viscosity of DNA solutions containing acridine dyes was enhanced, while the sedimentation coefficient of the DNA was diminished,⁽¹⁾ and by small angle x-ray scattering studies of the DNA-proflavine complex.⁽⁷⁾

Although the experiments reported by Lerman^(1, 6, 7) are indeed suggestive of intercalation, a number of important points are still unresolved. In particular, no accurate information has been reported for the stoichiometry of the complex, nor are any estimates available for the binding constants of the DNA complexes. Furthermore, the interpretation of the hydrodynamic and x-ray data depends upon the choice of a suitable model for the DNA molecule in solution, a largely unresolved question in itself.

The primary objectives of this study were, therefore, 1) to confirm the existence of a specific molecular interaction between DNA and acridine dye molecules using a different technique from that of Lerman, and 2) to obtain quantitative data for the complexing reaction.

PROGRESS REPORT

The system chosen for initial study was acridine-DNA and the technique used for studying the binding interaction was continuous-wave high-resolution nuclear magnetic resonance spectroscopy. This technique has a distinct advantage over other methods since the parameters obtained are directly related to the electronic environment and motional behavior of the nuclei in the molecule. Hence, any complexation of the acridine by DNA will necessarily result in changes in the electron distribution and molecular motion, which will be reflected in the high-resolution proton spectrum. Changes in molecular mobility, i.e., a decrease in the rotational and translational motion, will be reflected

a characteristic linewidth and intensity change⁽⁸⁾ in the acridine spectrum and will provide a sensitive test of the binding hypothesis. Any alteration in electron

distribution, on the other hand, will lead to chemical shift changes which can be analyzed to yield information about the stoichiometry and equilibrium constant for the binding reaction.⁽⁹⁾

In the work completed thus far the high-resolution proton spectra have been measured at room temperature for acridine and acridine-DNA solutions over a wide range of concentration and in varying solvent conditions. The spectra were recorded with a Varian DA60 spectrometer equipped with a field-lock accessory. Because of the low concentrations of acridine, 10^{-2} – 10^{-4} M/liter, the technique of repetitive scanning was used in order to improve the signal-to-noise ratio. The data from each scan were stored in a C-1024 time-averaging computer and the averaged spectrum was then recorded at the end of an experiment. For example, a 5×10^{-4} M acridine solution in 95% D₂O-5% methanol was scanned 4000 times (approximately 96 hours) to yield a spectrum showing distinguishable fine structure with a signal-to-noise ratio of 2:1.

As an illustration of the technique the results obtained for 10^{-3} M acridine, and 10^{-3} M acridine-0.5% DNA in 1:1 D₂O-CH₃OH solutions at room temperature are shown in Figures 178A and 178B, respectively. The spectra were recorded for each solution after 1100 scans; the resolution of the spectrometer was unchanged over the period required for the two experiments. It is evident that the spectrum for 10^{-3} M acridine is well resolved with a linewidth, ~ 0.5 cps, limited by the resolution of the spectrometer. By comparison the spectrum for 10^{-3} M acridine in a solution containing calf thymus DNA (highly polymerized) shows a very marked broadening of *all* of the lines for

FIG. 178.—A. Proton magnetic resonance spectrum for 10^{-3} M acridine in 1:1 D₂O-CH₃OH after 1100 scans. B. Proton magnetic resonance spectrum for 10^{-3} M acridine in 1:1 D₂O-CH₃OH; DNA concentration, 0.5%; number of scans, 1100.

acridine. The broadening is accompanied by a decrease in the signal intensity as is apparent from the low signal-to-noise ratio in Figure 178B. The line-broadening effects become even more pronounced at lower acridine concentrations and for $\sim 10^{-4}$ M acridine-0.5% DNA solutions no signals are observable for acridine after 4100 scans.

The line-broadening and intensity changes can be interpreted in two possible ways:

1) The acridine is bound irrotationally to the DNA and, hence, moves with the much longer correlation time associated with the DNA molecule. This would have the effect of decreasing T_2 (and T_1) and, therefore, increasing the linewidth.

2) The acridine is exchanging between two sites, i.e., acridine-DNA $\rightarrow$ acridine (free) at a rate comparable to the chemical shift difference between bound and free molecules.

Although further work is essential in order to establish which mechanism predominates it is apparent, nevertheless, that the present results can only be interpreted in terms of a molecular interaction between acridine and DNA. The NMR method, therefore, pro-

vides an independent confirmation of the viscosity and x-ray studies reported by Lerman.

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STABILITY OF THE SECONDARY STRUCTURE OF NUCLEIC ACIDS

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PURPOSE AND METHODS

As a means of interpreting the ionic equilibria of the purine and pyrimidine bases in DNA and their effect on the helix-to-coil-transition in DNA, an investigation of the thermodynamics of ionization of guanosine has been carried out. The pK'_a for the ionization of guanosine has been determined spectrophotometrically as a function of temperature, and potentiometrically as a function of temperature, concentration and solvent (H_2O and D_2O), at an ionic strength of 0.100 M NaCl. pK'_a refers to the equilibrium constant for the reaction: $BH^+ \rightleftharpoons B + H^+$, where B is guanosine.

PROGRESS REPORT

The results for guanosine at 25°C are tabulated in Table 91. A comparison of the heat of ionization of 2.86 kcal/mole derived from the temperature coefficient of the dissociation constant, and that from a calorimetric study,⁽¹⁾ of 0.99 kcal/mole indicates that the dissociation of a proton from the guanosine cation is in all probability, a complex reaction. Subsequent equilibria that might account for this large difference in the heat of ionization would conceivably involve tautomerism

of the $-N_1H$ proton of guanine, conformation of the ribose group, or conformation of the glycosidic link between the guanine and ribose.

It is seen in Table 91 that replacement of H_2O as solvent by D_2O leads to an increase in pK'_a of 0.42 in agreement with what is generally observed for isotopic substitution in low molecular weight acids.⁽²⁾ Although only limited data are available at the moment for the temperature coefficient of the equilibrium constant in D_2O , it appears to be unaffected by isotopic substitution, implying that of the possible intramolecular interactions that accompany the dissociation of a proton from the guanosine cation, only those that do not directly involve a proton need be considered. Because the heat of ionization is unaffected by isotopic exchange it may be concluded that the accompanying increase in pK'_a is largely entropic in origin.

Potentiometric determinations of the pK'_a of guanosine are carried out at concentrations that are an order of magnitude greater than are employed in the spectrophotometric method. Under these conditions a pronounced dependence of pK'_a upon concentration is noted; this concentration dependence is in turn a

TABLE 91

Method	Solvent	pK'_a	ΔF_{ioniz} , kcal/ mole	ΔH_{ioniz} , kcal/ mole	ΔS_{ioniz} , cal/deg/ mole
A. Spectrophotometric, 3×10^{-4} M guanosine	H ₂ O	2.18 ± 0.01	2.98	2.86 ± 0.27	-4.0
B. Potentiometric, infinite dilution	H ₂ O	2.06	—	—	—
	D ₂ O	2.48	—	—	—

function of solvent composition and temperature. The increase in pK'_a that is observed is consistent with the formation of complexes between neutral and cationic guanosine, the extent of polymerization increasing in with temperature in H₂O. The extent of polymerization in D₂O is smaller at all temperatures.

Calorimetric observations of the protonation of

native DNA have shown ΔH_{ioniz} to be zero,⁽³⁾ whereas the ΔH_{ioniz} for the various nucleosides varies from 1 to 4.5 kcal/mole.⁽¹⁾ Such an observation is consistent with base pair interaction being enhanced by protonation of a neighboring purine ring, such an interaction being endothermic in the case of association polymers of guanosine.

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RBE STUDIES: THE USE OF MOUSE TESTIS WEIGHT AS A SENSITIVE BIOLOGICAL INDICATOR

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PURPOSE AND METHODS

We plan to study, in mammalian tissues, the relative biological effectiveness (RBE) of monoenergetic neutrons with energies in the range 0.1 to 15.0 MeV. The Division requires, for this purpose, a high intensity source of such neutrons, but at present only existing Van de Graaf accelerators in other Divisions of the Laboratory are available, and these provide dose rates of a few rads per minute.

The testis weight response in male mice has been chosen as an indicator in these studies for the following reasons. The time-course of testis weight changes following irradiation is well known. After moderate doses, it falls to a minimum at about 24 days post-irradiation and recovers in about 42 days. This effect is believed to be a consequence of damage to a single cell type, the type A spermatogonium. Radiosensitivity is great, though it differs in various mouse strains. In the CBA mouse, for example, the mean testis weight falls to 60% of that of unirradiated controls after 100 R exposure to x-rays.⁽¹⁾ The testis is relatively insensitive to other stresses and is unaffected by irradiation of the rest of the body.⁽²⁾ It is nominally situated

mm beneath the skin surface, providing adequate protection build up for neutrons of 15-MeV energy. Attenuation and energy degradation of the primary neutrons are not severe. Accurate location of the organs

within a few centimeters of the neutron source by constraint of the mouse in an exposure tube is fairly simple, and avoids the necessity for collimation.

PROGRESS REPORT

Three types of mice have been studied to determine the normality of distribution in average testis weight (average of the weight of the two testicles in each animal). CF $\times$ 1 Anl mice (random-bred and randomly grouped, 270 animals, age 9-14 weeks) exhibited a standard deviation for a single observation of 9.8%. In the case of CF $\times$ 1 Anl siblings (random-bred, grouped by litter, 177 animals, age 13 weeks) the standard deviation for a single observation was 6.27%. In comparison, C57BL/6J mice (inbred strain, randomly grouped, 217 animals, age 11 weeks) the standard deviation for a single observation was 8.4%. Of these 3 types, the CF $\times$ 1 Anl mice (caged by litters and with litter-made controls) appear the best choice. The reduced standard deviation should permit a useful reduction in the number of experimental exposures required to attain the desired goal of standard error in the mean.

In the CF $\times$ 1 Anl mice, the variation in body weight, average testis weight, and ratio of left/right testis weights, and testis weights to age has been investigated in the range of 5-14 weeks. Body weight increases

linearly with age from 6-14 weeks, but average testis weight remains fairly constant after age 9 weeks. No evidence was obtained to support the practice of using extreme individual body weights as a basis for rejecting testis-weight data.

The testis-weight ratio was fairly constant at 0.936 with a standard deviation of 3.4%. Some 15% of the animals exhibited an inverse ratio. These data were inverted and were found to fit the same normal distribution curve. In an attempt to use these promising statistics, an experiment was carried out in which the left testicles were removed, under anesthesia, and weighed. Two days later one-half of the animals were irradiated with 100 R x-rays (HVL 1.6 mm Cu). The time course of weight change in the residual testicle of both irradiated and control animals was followed by serial sacrifice at 2, 3, 4, 5, and 6 weeks postirradiation. The weight of the residual testicle was expressed as a ratio to that of the previously removed, or auto-control testicle. Postsurgical hypertrophy of the residual testicle was marked and quite variable. Interpretation of the data was also confused by the 15% of animals having the weight ratio inverted, for which no reliable correction has been devised.

CONCLUSIONS

C57BL/6J mice were found to have a testis-weight ratio of 1.00 and to be free of the ratio inversion effect, but for the present this line of investigation will not be pressed further.

The time course of average testis weight following a single exposure to 100 R x-rays has been determined in CF $\times$ 1 Anl mice as follows: 2 weeks, 85% of control; 3 weeks, 71%; 4 weeks, 73%, and 5 weeks, 94%. This pattern follows that established for other strains. The percent weight loss is 70% of that observed in CBA mice for the same dose.⁽¹⁾

The response of average testis weight to x-ray exposures of 45, 65, 100, and 150 R has been established. A linear regression line is obtained (percent testis weight/log dose) and will serve as a base line for future studies.

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AN AUTOMATIC ADAPTER FOR MANUAL POWER CONTROLLERS

Norman A. Frigerio

PURPOSE AND METHODS

Many laboratory devices require slowly-increasing sources of power (high-speed centrifuge motors, large vacuum-distillation heaters, shunt-wound motors, etc.). Power is ordinarily applied by manual adjustment of a variable autotransformer.

This manual control suffers from three disadvantages, even if a timer is used to cut off power at some predetermined time: 1) Manual control is seldom smooth or reproducible so that an erratic element is introduced into experiments. 2) If the power control is not re-zeroed before beginning another run, as often happens, either a fuse is blown or the drive device is damaged. 3) Manual control is generally tedious and, in incubators, refrigerated or radiation environments may range from uncomfortable to unsafe. Such discomfort tends to exaggerate 1) and 2).

Our frequent encounter with such situations led us to design a simple, low-cost control for slowly raising device power level to a fixed value, holding it there for a predetermined time, and then returning the control

to zero. These controls have proved particularly useful for slow distillations, for high-speed centrifugation with older-model controlled-speed centrifuges, and for devices used in radiation fields.

PROGRESS REPORT

The design of the device is shown in Figure 179. Physically, it consists of a small, reversible, ac, synchronous motor equipped with clamps, switches and a shaft adapter. It can be clamped, in place of the knob, on any manual control. We used a 1/7 rpm, 8-watt unit which delivers 80 oz-in. of torque,* about the same as a human hand. Similar motors are available at low cost and over a wide range of angular velocities.

Although our shaft adapter was machined for 1/8" to 1/4", any size up to 1/2" could be driven by the 1/8" motor shaft. Two simple lugs were screwed to the shaft adapter for activation of the limit microswitches.

For installation, the controller knob is removed,

* Bristol Motor Co., Old Saybrook, Conn., Model 830-1/7-19-2AF.

FIG. 179.—Automatic power controller

placed with the open end of the shaft adapter, and the unit is clamped firmly in place with the counter clockwise limit switch at the position corresponding to zero power. The clockwise switch is then clamped to the position corresponding to the desired maximum power level.

When the SPST timer closes, the relay applies power through 3 to the motor, which then rotates steadily clockwise, at a rate determined by its design rpm, closing the counter clockwise limit switch at the same time. When the desired power is reached, the clockwise limit microswitch opens and the motor rests. At the end of the run the timer opens and the relay returns power through 4 to the motor, which then rotates counter clockwise. Figure 179 shows this condition. When zero power is reached the clockwise switch is closed, the counter clockwise switch opens and the system stops.

If a DPDT timer is available, the relay may be

eliminated, power applied to 2-3 for ON, and to 1-4 for OFF.

Although the motor used here has a single speed for both directions, similar motors are available,* at higher cost, which permit independent selection of speeds. The unit can be constructed in about 3 man hours with simple hand tools. The two that were constructed have operated for 7 years without malfunction, (one at 1°C and 100% relative humidity), so that they may be regarded as fairly reliable devices.

CONCLUSIONS

A simple, inexpensive torque-motor device is described for applying automatic control to manual power controllers. The device has proved particularly useful where adapted to radiation environments, long distillations and to the simpler models of high-speed centrifuges.

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Plant Radiobiology

X-RAY EFFECTS ON THE FORMATION OF FLOWER PRIMORDIA IN XANTHIUM

William Chorney

PURPOSE AND METHODS

Xanthium is a short-day plant whose flowering is controlled by photoperiod.⁽¹⁾ It is one of the most sensitive of the short-day species in that, under optimum conditions, it can be induced to form flower primordia by subjecting but a single leaf to one inductive cycle. The inductive cycle that is most efficient consists of two periods of high light, one preceding and the other following the inductive dark period.⁽²⁾ The most sensitive leaf for induction is the leaf that is expanding most rapidly.⁽³⁾ This is normally the third leaf out of the bud longer than one centimeter. With this system the effects of x-rays on the formation of flower primordia can be studied when the x-rays are applied during various phases of the inductive cycle.

Xanthium plants were maintained in a vegetative state by growing them on an 18-hour photoperiod in the greenhouse. When the plants were 4 to 5 weeks old from sowing, they were selected for uniformity of leaf size at the 6th node. The plants were trimmed to a single leaf; all leaves longer than 1 cm were removed except the leaf at the 6th node which was one-half to three-quarters expanded. Selection and trimming of plants was carried out during the light period preceding the inductive dark period. X-rays were applied only to the inducing leaf. The shoot tip was shielded from radiation with at least ¼ inch of lead. The exposure rate was 50 R/min from a General Electric Maximar x-ray machine operated at 250 kV at 15 mamp with no added filtration. After the inductive night the plants were given high light of 1200–1500 fc. under a fluorescent panel for 8 hours, unless otherwise noted, before being returned to the 18-hour photoperiod in the greenhouse. When plants were treated with low light they received 40 fc. incandescent light. Ten days later the shoot terminal meristem was examined under the dissecting microscope to determine the amount of floral development. Flowering was scored according to Salisbury's scheme.⁽³⁾

PROGRESS REPORT

A. The Effects of X-rays when Applied Prior to the Inductive Night

The plants were exposed to various amounts of x-rays up to 5.0 kR prior to the 9¼-hour inductive night. The results are plotted in Figure 180. Control plants reached Stage 2 of floral development. With as little as 250 R of x-rays, stimulation of flowering was obtained. Saturation of the stimulatory effect was observed at about 1 to 2 kR.

In another experiment, plants were given 1.0 kR of x-rays and the leaves of the plants were treated with various concentrations of gibberellic acid (GA_3) in 0.1% Tween-20, using a 12-second dip, prior to the 9¼-hour inductive night. The results are shown in Table 92. Control plants, no x-rays or GA_3 , reached a flowering stage of 3.1, while x-rayed plants attained a 5.4 stage. Plants treated with various concentrations of GA_3 in addition to x-rays showed even a greater degree of flowering than those receiving x-rays alone. GA_3 in concentrations from 1 to 50 mg/l had no effect on flowering when applied to unirradiated plants.

B. X-ray and Light Effects when Applied Following the Inductive Dark Period.

It has been reported that 3.0 kR of x-rays applied to *Xanthium* within the first light period following the inductive dark period suppressed flowering.⁽⁴⁾ If application of x-rays was delayed for 24 hours there was no effect on flowering. A number of experiments were run to determine more precisely the time period in which x-rays were effective in suppressing flowering when the x-rays were applied following the inductive dark period. In all the following experiments, the plants were subjected to an 11.5-hour night. After the dark period, the plants were placed in high light and x-irradiated with 3.0 kR at various intervals up to 25 hours. In another series of experiments, the effect of high light on flowering was determined. In these experiments, the

FIG. 180.—X-ray effects on flowering in *Xanthium* applied prior to the inductive night

plants were exposed to various periods of high light and then placed in low light for the remainder of the 14.5-hour postinductive light period. In a third series of experiments, the translocation curve for the movement of the flowering principles out of the leaf was determined.^(2, 5) This was done by removing leaves from plants kept under high light at intervals up to 25 hours. The results of these three sets of experiments are plotted in Figure 181.

After 5 to 10 hours in high light, the x-rays are no longer effective in suppressing flowering. Flowering is increased with increased exposure to high light, saturating at about 4 to 5 hours. The translocation curve shows that most of the flowering principle is still in the leaf after 10 hours in high light.

Another experiment was conducted in which the plants were given low light for varying times up to 7 hours before giving high light for the remainder of the postinductive 14.5-hour light period. In addition, other lots of plants were x-irradiated with 3.0 kR after periods of low light up to 4.0 hours. These results are shown in Figure 182. Increased periods of low light decreased the flowering response. Subjecting plants to low light for 7 hours prevented flowering. X-rays given in conjunction with one hour of low light markedly suppressed flowering. After 4 hours of low light 3.0 kR of x-rays completely inhibited flowering.

How can the stimulatory effects of x-rays applied prior to the inductive night, and the enhancement of this effect by GA₃, be explained? A number of investigators have found that application of indoleacetic acid (IAA) to the inducing leaf before, during, or shortly after the inductive dark period suppressed flowering in *Xanthium*.^(2, 3, 6, 7) Gordon has shown that x-rays applied to *Xanthium* leaves will lower the auxin

TABLE 92. EFFECTS OF X-RAYS AND GIBBERELIC ACID APPLIED PRIOR TO THE INDUCTIVE NIGHT ON FLOWERING IN *Xanthium*

x-ray, kR	Gibberellic acid, mg/l	Flowering stages, $\pm$ S.E.*
0.0	0.0	3.1 $\pm$ 0.3
1.0	0.0	5.4 $\pm$ 0.2 ^(a)
1.0	1.0	6.7 $\pm$ 0.3 ^(a, b)
1.0	10.0	7.0 $\pm$ 0.2 ^(a, b)
1.0	50.0	7.8 $\pm$ 0.2 ^(a, b)
0.0	1.0	3.0 $\pm$ 0.2 ^(c)
0.0	10.0	2.6 $\pm$ 0.4 ^(c)
0.0	50.0	3.0 $\pm$ 0.4 ^(c)

^(a) Significantly different from control at 1% level.

^(b) Significantly different from x-ray at 1% level.

^(c) Not significantly different from control, 5% level.

* S.E., standard error of mean.

level.⁽⁸⁾ It is quite possible that the endogenous auxin level in *Xanthium* is of sufficient concentration to be inhibitory towards flowering; therefore, reducing the auxin level with x-rays results in a stimulation of flowering. Chailakian has proposed that gibberellins are necessary for flowering to occur.⁽⁹⁾ He has stated that for short-day type plants growing on long photoperiods the GA requirement is not limiting for the flowering process. It is quite possible that x-rays in addition to lowering the auxin level in plants may also lower the GA level to a point where GA does become limiting. Therefore, application of GA will lead to a more advanced flowering than x-rays alone.

Lockhardt and Hammer have proposed a hypothesis for the events that occur in the postinductive light period.^(2, 7) Their results state that if the induced leaf is

FIG. 181.—Effect of x-rays, high light and leaf removal on flowering in *Xanthium*

FIG. 182.—Effect of low light, 40 fc, and x-rays, 3.0 kR on flowering in *Xanthium* applied after the inductive night.

given a brief exposure to high light, 10 minutes at 1000 fc., and followed by a second dark period of 5 hours at elevated temperatures or with the addition of IAA that the leaf is capable of destroying the flowering principles produced by the inductive dark period. However, if the leaf is subjected to 5 hours of high light the flowering principle is stabilized and can, on being translocated to shoot meristems, initiate the flowering process. Results reported in this manuscript are in agreement with their results except that under the conditions of the present experiments, destruction of flowering principle was obtained under low light at normal temperatures without the addition of exogenous IAA. One conclusion that can be drawn from the results obtained by x-irradiating in the postinductive light period is that the x-rays must be applied before

stabilization of the flowering principle has occurred in order to suppress flowering.

Since the results from low light and low light plus x-ray treatments were not additive, it is likely that the pathways involved in obtaining the suppression of flowering under these environmental conditions are different.

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CORRELATIVE ACTION OF THE GAMMA-IRRADIATED LEAF ON ROOT INITIATION IN COLEUS

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PURPOSE AND METHODS

When the *Coleus* leaf is excised and its petiole placed in water, adventitious roots are formed at the petiole base within 5 to 6 days after excision. The formation of these roots is delayed by exposing only the leaf-blade to x- or γ radiation, the degree of inhibition being related to the dose. From an early study⁽¹⁾ it is known that the leaf blade limits the formation of roots in subjacent tissues by the rate at which it supplies carbohydrate, nitrogenous factors, and auxin. We are curious about the relative radiosensitivity of these limiting factors, and are determining if their concentrations are altered in the petiole beneath the irradiated blade. The radiation protocol is attractively simple for the study of correlative action in plant morphogenesis: the locus of radiation absorption is separated from the site of differentiation.

Gamma irradiation of the leaf with exposures that halve root emergences in the shielded petiole base has no material effect on the mono- and polysaccharides, or the "soluble" and "insoluble" nitrogen levels in the petiole base.⁽²⁾ However, the RNA content in these

petiole tissues is lowered significantly within 12 hours by irradiation. The depression of RNA content is more pronounced 24 hours later, before histological differences are observed microscopically. Irradiation of the leaf has no material effect on the extent of incorporation of labeled uracil into the RNA of the petiole.⁽²⁾ Thus the change in RNA level appears not to be a consequence of curtailed RNA synthesis. The alternative is a radiation-induced increase in RNA degradation in the petiole base. An enhanced loss of RNA could come about by either a decrease in resistance to hydrolysis by RNAase, or an increase in activity of RNAase. The present report describes the effect of irradiating the blade on the RNAase activity in the petiole base.

Yeast RNA was used as a substrate. Soluble nucleotides were measured spectrophotometrically after uranyl-acetate precipitation of nucleic acid.⁽³⁾ Leaves were arranged along an isodose arc centering on a 6,000-curie, Co^{60} source; the petioles, with their bases in water, were shielded by 4 inches of lead. The exposure rate was ca. 250 R/min.

FIG. 183.—Hydrolysis of yeast RNA by *Coleus* petiole homogenate at various pH's. Time, 45 minutes; temperature, 30°C; RNA concentration, 5 mg/ml.

FIG. 184.—Effect of γ irradiation of the *Coleus* leaf blade on the observed (solid lines) and specific (broken lines) RNAase activities of the shielded petiole base. Parentheses indicate number of replicates, 20 leaves per replicate.

PROGRESS REPORT

We find that the cell-free homogenate of the base of the *Coleus* petiole has, as do the corn seedling⁽³⁾ and the rodent thymus,⁽⁴⁾ two pH optima (Figure 183). The petiolar hydrolase has peak activities at pH ~ 4.7 and 7.7, whereas the corn RNAase peaks at pH 5.0 and 6.2. The two pH maxima of the thymus are at 6.0 and 8.0.

Further work was restricted to the activity at pH 4.7, since the alkaline RNAase activity was difficult to reproduce. From preliminary studies of hydrolysis rates, a 45-minute incubation time with a 1:2 petiole homogenate in water, temperature 30°C, and RNA concentration of 5 mg/ml, yielded a nearly linear reaction rate. Using these assay conditions to index enzyme activity, the effect of γ irradiation of the leaf blade on the RNAase activity in the shielded petiole base was determined (Figure 184). There was no effect discernable 6 hours after irradiation, but at 14, 24, and 40 hours after, both the observed and specific RNAase activities were higher in the petioles under irradiated blades than those in nonirradiated but otherwise identi-

cally treated leaves (analysis of variance, $p < 0.01$). The ratio of activities of the irradiated to control leaves appears to be higher at 15 and 25 hours than at 40 hours after irradiation.

We infer that the loss of RNA that occurs in the petiole base soon after irradiation of the lamina occurs, at least in part, from a hormonal activation of ribonuclease activity in the base. This does not exclude the possibility that either concomitant with or causally related to the activation of the hydrolase is an increased lability of the petiolar RNA itself.

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EFFECT OF LIGHT ON THE METABOLIC RESPONSES OF SQUASH SEEDLINGS

Kenneth Surrey and Edward Barr

PURPOSE AND METHODS

Growth and development of plants is primarily controlled by the two well-established photoreactive systems: Systems related to the development and functions of the photosynthetic apparatus, and photomorphogenic reactions controlled by the phytochrome system.

This report deals with the light-induced responses in lipoxidase of etiolated squash seedlings and their relationships to the known photoresponsive systems. To accomplish this objective, studies were conducted to observe whether lipoxidase is affected directly by light or if it is influenced by the chlorophyll synthesis, and to observe if any causal relationship exists between lipoxidase responses and the presence of phytochrome.

The 96-hour dark-grown squash seedlings (*Cucurbita moscata*) were exposed to various light intensities (from 0–2000 fc) for different time periods up to 24 hours. Following light exposures, the seedlings were returned to darkness. Regardless of the duration of light exposure, the first sample was taken on the fifth day and additional samples were taken every 24 hours until the eighth day from the start of hydration.

Twenty cotyledons from each batch of the seedlings were ground in sand; the pigment was extracted repeatedly with diethyl ether until the paste was dehydrated and until the washings as well as the residue were colorless. The ether extracts were pooled, dehydrated with anhydrous Na_2SO_4 and the total chlorophyll of the extract was determined spectro-

Fig. 185.—Lipoxidase activity curves of cotyledons obtained from the irradiated and nonirradiated squash seedlings. Four-day c-grown seedlings were exposed to various light intensities for 3 hours and then returned to darkness. Sampling was repeated after every 24 hours from the fifth to the eighth day. The rates of exponential decline in enzyme activity given on the respective curves, are the decay constants (k) of the first-order activity curves minimized by least squares. These exponential rate constants were calculated from the fifth through the eighth day (see Figure 186 for decay constants of other light exposures).

FIG. 186.—The effect of light on the decay constants for lipoxidase activity. Each datum represents a decay rate (k) for the seedlings exposed to a fixed irradiance for a fixed time (cf. Figure 185), and each curve is for the decay constants of the seedlings given indicated light exposures for a fixed time. Exposure time for each curve is given on the right side. The rate of decay constants (K), as a function of log-light intensities for a given exposure time, is indicated on the respective curve.

TABLE 93. EFFECT OF LIGHT ON CHLOROPHYLL FORMATION AND LIPOXIDASE ACTIVITY IN THE COTYLEDONS OF SQUASH SEEDLINGS

Light intensity, foot candles	Exposure time, hours			
	0.75	1.50	0.75	1.50
	Decay constants/day, k		μg Chlorophyll/cotyledon	
	Rate of decay constants /log increment fc, K		μg Chlorophyll/cotyledon per log increment fc (b)	
31.25	-0.652	-0.794	1.08	1.66
62.5	-0.704	-0.778	1.09	1.72
125	-0.770	-0.863	1.05	1.79
250	-0.793	-0.820	0.96	1.85
500	-0.778	-0.860	1.38	1.82
1000	-0.813	-0.854	1.43	1.99
2000	-0.821	-0.887	1.27	1.92
	-0.037 $\pm$ 0.021	-0.023 $\pm$ 0.022	0.081 $\pm$ 0.042	0.060 $\pm$ 0.028

photometrically.⁽¹⁾ Lipoxidase activity of the ether-free residue was determined spectrophotometrically.^(2, 3) For long-wave ultraviolet radiation, a Black-Ray lamp transmitting over a range of 3075-4047A was used. The radiation intensity was $750 \mu\text{W}/\text{cm}^2$ at the seedling height. A mineral light lamp was employed for short-wave (2537A) UV irradiation emitting $260 \mu\text{W}/\text{cm}^2$ flux at the seedling height. For prolonged red (660 m μ) and far-red (735 m μ) radiation, monochromators fitted with interference filters were used.⁽⁴⁾ The incident energies at both these wavelengths were equalized to about $40 \mu\text{W}/\text{cm}^2$. Phytochrome in the etiolated seedling was detected *in vivo* with a dual-wavelength difference photometer.⁽⁵⁾

PROGRESS REPORT

Lipoxidase activity in the cotyledons of squash seedlings begins at about 48 hours from the start of hydration, reaches a peak at about the fifth day and then declines to its lowest level of activity on the eighth day (See Figure 185; dark). The lipoxidase responses shown in Figure 185 indicated that the zero-

FIG. 187.—The amount of chlorophyll synthesized in the cotyledons of squash seedlings exposed to various light intensities (log scale). Each datum represents an average value of pigment produced in the cotyledons of four-day etiolated seedlings given a single exposure to white light for a fixed time, and each curve is for the seedlings exposed to indicated irradiance for a given exposure time. Time of exposure for each curve is shown on the right side. The rate of chlorophyll produced (b), as a function of log-light intensities is given on the respective curve.

order activity rates of the enzyme decay exponentially and that the decay constants (k) increase with the increase in light intensity.

In initial experiments, four-day etiolated seedlings were exposed continuously to various light intensities (as shown in Figure 186). They were then periodically assayed for lipoxidase activity. The rate of enzyme depletion was accelerated to such an extent that under high light exposures (e.g., 500 fc and above) the activity rates reached the lowest levels within 48 to 60 hours of illumination. Therefore, the light treatments were restricted to 24 hours and below.

The decay constants (k) for lipoxidase activity of the seedlings exposed for 3, 6, 12 and 24 hours, to a geometric increase in light intensities, are shown in

Figure 186. Not only are the decay constants increased linearly with the increase in radiant flux, but the rate of increase became more pronounced when the seedlings were treated at geometric increments in exposure time. Likewise, reducing the exposure time from 3 to 1.5 and then to 0.75 hours caused a relative decrease in the rate constants (Table 93, left half); a decrease that tends to approach the decay constant for the dark control (See Figure 186).

To facilitate the presentation of these data, the rate of change in rate constants, as a function of irradiance, were calculated (K) and are shown on the respective curve (Figure 186; see also Table 93, left side). The curve for the change in rate constants (K) in relation to the exposure time is shown in Figure 188, indicating

FIG. 188.—Combined data on Figures 186 and 187 together with the responses obtained by the shorter light exposures (Table 95) showing the effect of various exposure times (log scale) on the rate of decay constants (K) and the rate of chlorophyll formation (b).

FIG. 189.—The effect of ultraviolet light on lipoxidase activity (●—●, left ordinate) and chlorophyll formation (x—x, right ordinate) as related to various exposure times (log scale). Top part depicts the responses with shortwave, and the lower part is for the seedlings exposed to the longwave ultraviolet light. For an explanation of the parameters indicated, see legends of Figures 185 and 187.

that the lipoxidase activity is decreased with an increase in light intensity, a decrease that is further accentuated with an increase in exposure time.

The chlorophyll content of the cotyledons, on the other hand, increased with the increase in radiant flux; no light-saturation stage was reached even when the seedlings were continuously irradiated from the fourth through the eighth day.

As mentioned for lipoxidase, the rates of chlorophyll formation as a function of irradiance, were calculated (b) and are indicated on the respective curve (Figure 187; see also Table 93, right side). The curve for the rates of pigment synthesis, in relation to the time of exposure is depicted in Figure 188. Chlorophyll synthesis proceeded at two different levels; a lag period extending up to about 4–5 hours and an accelerated stage beyond five hours. In the former stage, especially for 0.75 and 1.5 hours of exposure, independent of the amount of light administered, the rate of chlorophyll synthesis remained nearly constant, whereas in the later stage a strict linearity was maintained with respect to the light intensities and the time.

The dose-response curves for pigment synthesis and enzyme disappearance (Figure 188) suggest an inverse relationship. This relationship was investigated by the use of ultraviolet and red, far-red irradiation.

Ultraviolet Irradiation

Rate constants (k) for lipoxidase activity and chlorophyll synthesis in the seedlings, exposed to a

fixed level of long-wave UV radiation, for varying times, are shown in the lower half of Figure 189. Qualitatively, the effect of long-wave UV irradiation can be compared with the photoresponses induced by the white light, with the exception that in the latter the decay constants consistently increased relative to the dark control (See Figure 186), whereas in the former, shorter exposures (3, 6 and 12 hours) resulted in rates that approached and even exceeded the decay constant for the unirradiated seedlings.

The decay curve for enzyme activity of the seedlings irradiated with the short waves of the UV light is shown in the top part of Figure 189. The decay constants decreased with each increment of irradiation time, indicating that the enzyme disappears even more slowly than in the unirradiated seedlings. At the same time the chlorophyll content (for 12 and 24-hour exposures) exceeded the amount synthesized in the seedlings given shorter exposures to white light (See Table 93). If chlorophyll synthesis were the causal response to effect enzyme depletion then the relationship between the curves for lipoxidase and pigment synthesis should have shown an inverse relation as depicted in Figure 188.

In view of the kinetics of enzyme responses coupled with the formation of chlorophyll, it appears unlikely that the lipoxidase metabolism was influenced by the pigment synthesis.

Red, Far-Red Irradiation

The results of four-day-old etiolated seedlings exposed to continuous red and far-red light are shown in Table 94. Red light produced chlorophyll and at the same time retarded the enzyme depletion. Far-red light, on the other hand, did not cause the pigment

TABLE 94. EFFECT OF CONTINUOUS RED AND FAR-RED IRRADIATION ON LIPOXIDASE ACTIVITY AND CHLOROPHYLL FORMATION IN THE COTYLEDONS OF DARK GROWN SQUASH SEEDLINGS

Sampling period, days	Red light (660 m μ)		Far-red light (735 m μ)	
	Units of lipoxidase/cotyledon per min	μ g Chlo-rophyll/cotyledon	Units of lipoxidase/cotyledon per min	μ g Chlo-rophyll/cotyledon
5	54.05	22.8	46.9	1.4
6	32.80	50.3	17.0	1.4
7	16.66	73.4	2.88	1.1
8	2.75	102.3	0.06	1.0
Decay constant/day				
	<i>k</i>		<i>k</i>	
	-0.96		-2.16	

TABLE 95. RELATIVE AMOUNT OF REVERSIBLE PHYTOCHROME IN DARK-GROWN SQUASH SEEDLINGS

Growth period, days	$\Delta(\Delta OD)$		
	Cotyledons	Hypocotyl	Roots
0*	0.002	—	—
Wet	0.004	—	—
1	0.005	—	—
2	0.009	0.004	
3	0.080	0.009	0.003
4	0.051	0.002	0.003
5	0.053	0.002	0.002
6	0.038	0.002	0.002

* Zero denotes dry seeds, and wet represents seeds soaked 6 hours.

synthesis but accelerated the rate of enzyme depletion that was twice as much as was observed for the seedlings exposed to red light—a clear indication that lipoxidase metabolism and chlorophyll synthesis are two separate light-induced responses and each response proceeds independently of the other.

Phytochrome Estimation in vivo

The relative amounts of red, far-red reversible phytochrome, present in various organs of the dark-grown squash seedlings, are shown in Table 95: The cotyledons contained the greatest amount and reached a maximum level on the third day and then declined thereafter. Pigment levels in the hypocotyls and roots were much lower than in the cotyledons and remained nearly constant throughout the growth period.

CONCLUSIONS

A) The enzyme lipoxidase decays in the dark (Figure 185, dark). The rate of decay was increased linearly with the geometric increase in light intensity (Figure 186) and became more pronounced with the increase in exposure time (Figure 188). Although enzyme was depleted with light intensity and time, continuous irradiation was not required for a maximum response. Seedlings given high light intensities (500 fc and above) showed close to a complete disappearance of enzyme within 48 to 60 hours of extended exposure.

B) Chlorophyll continued to increase with an increase in light and exposure time (Figure 188). No light-saturation level was reached even when the seedlings were exposed to light for four days after the initial four-day dark period.

C) Ultraviolet and red, far-red irradiation provided

no adequate basis for any causal interdependence between lipoxidase disappearance and chlorophyll formation.

D) Lipoxidase responsivity depended solely upon high light intensities far beyond the range of irradiance at which phytochrome or phytochrome-dependent reactions are saturated.

E) Even though phytochrome was present in the cotyledons, its presence did not seem to have any physiological significance in the light-activated disappearance of the enzyme. This conclusion was based on the fact that there was no evidence indicating any light-saturation effect because the curve for the change in rate constants (Figure 188) continues to approach zero, a level representing dark control.

F) The study with white light suggested that lipoxidase metabolism is neither related to the chlorophyll formation, nor is it influenced by the presence of phytochrome. Enzyme responses like those shown here may be considered to represent a separate class of photoreactions occupying a position between continuous light-requiring systems that support the development and function of the photosynthetic apparatus and systems responding to discontinuous low

light-requiring photomorphogenic responses controlled by photochrome.

G) The disappearance of lipoxidase in light corresponded closely to the enlargement of the cotyledons, a phenomenon known to be brought about by the high-energy reaction.⁽⁶⁾ Lipoxidase metabolism may be considered a biochemical index for the morphological developments controlled by the high radiant energy.

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ACTION SPECTRUM FOR THE PHOTOLYSIS OF MYXOCOCCUS XANTHUS

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PURPOSE AND METHODS

Preliminary experiments on the photolysis of *Myxococcus xanthus* with broad-band filters indicated that lysis efficiency was wavelength dependent. As the first step in identification of the photosensitizer, a more refined relative spectral response has been determined with the Argonne Biological Spectrograph.

M. xanthus was cultured in low Mg-CT medium⁽¹⁾ in the dark on a rotary shaker at 30°C. Cells, approximately 36 hours into stationary phase, were exposed to a 1-m μ -wide band of light at intervals of 10 m μ between 350 and 700 m μ ; 5-m μ intervals were used to define better the spectral regions of lysis.

Prior to exposure to light, cells were eluted 1:20 in fresh low Mg-CT and distributed in 2.5-ml samples in 3-ml Pyrex cuvettes. The cuvettes were inserted into rigid holders at various wavelengths. During illumination cells were aerated and agitated by bubbling washed, humidified air through each suspension. Photolysis was assayed by periodic counts of surviving

cells with a Petroff-Hauser counting chamber. The data were programmed in Fortran, analyzed and fitted using the IBM 1620.⁽²⁾ Variations of photosensitivity with age of cells in different experiments were compensated by correcting the slopes of the survival curves to reference sensitivities at 425 m μ .

FIG. 190.—Photolysis of *Myxococcus xanthus* with monochromatic light at several wavelengths.

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FIG. 191.—Top, cumulative energy dosage at each wavelength; bottom, action spectrum of photolysis of *Myxococcus xanthus*

PROGRESS REPORT

Five major experiments were carried out in spectral regions of photolysis; each experiment tested 5 to 10 wavelengths. Other apparently nonphotolytic wavelengths were studied in minor experiments.

As an example, the results of irradiation with 5 wavelengths run simultaneously in one experiment are presented in Figure 190. The logarithm of surviving cells is plotted against radiant density. The points represent total cell counts and are best described by a "multi-hit" curve with the relationship $S = 1 - (1 - e^{-kD})^n$, where S is the standardized response (fraction of survivors) to a dose D , and k is a constant, describing the slope of the curve.⁽³⁾ n , also a constant, has been interpreted as the number of "hits" necessary to bring about the death of cells exposed to ionizing radiation, and analogously, might here reflect the minimum number of photons required for lysis at a given wavelength.

The slope of the survival curve was used as a measurement of efficacy of each wavelength in inducing photolysis. The k values are plotted against wavelength in the lower part of Figure 191, which depicts the photolysis action spectrum. High k values (high rate of lysis) are plotted as peaks on the action spectrum; zero k values are plotted as troughs. The points plotted in the upper portion of Figure 191 give

the cumulative energy delivered to the cuvette at the termination of the experiment. At wavelengths above 550 mμ only high radiant densities elicited any response; these resulted in extended lag periods and low rates of photolysis. In contrast, photolysis at 410 mμ was essentially complete after a relatively low radiant density, with a virtually exponential survival curve. Other points showing no or low lysis might be spurious since it was difficult to attain high total energies at the longer wavelengths; the carbon arc had relatively low emission in this spectral region. With extended periods of irradiation, problems of cell clumping and evaporation of medium were encountered.

The action spectrum depicted in Figure 191 has a striking similarity to the generalized absorption spectrum for porphyrins.⁽⁴⁾

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Growth and Development of Plants in Compensated Gravitational, Magnetic, and Electrical Fields

ON THE FORCE REQUIRED TO ALTER GROWTH OF COMPENSATED OAT SEEDLINGS*

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PURPOSE AND METHODS

We are using several approaches in this laboratory to characterize the magnitude of force required to produce geomorphic changes in the plant. Such information bears on the nature of the gravitational sensor, and is directly pertinent to the programming of biosatellite motion. The present approach is based on our observations that the extent of elongation of compensated roots and shoots is dependent upon orientation when the plant is grown in a centrifugal field. This report describes the effect of acropetal and basipetal centrifugal force on the growth of oat seedlings developing in a 2π compensator,⁽¹⁾ a modified clinostat that nullifies the di-

rectional component of the gravity vector while enabling the imposition of various centrifugal forces in direction normal to that vector.

Seeds of *Avena sativa* (Victory I, Svalöf) were hydrated and planted on an agar medium in plastic vials. The seeds were oriented so that when the vials were attached horizontally to the 2π compensator, the embryonic shoot apex was directed either centrifugally and the radicle centripetally, or the reverse. Thus the longitudinal axes of the emerging organs were oriented along the horizontal axis of rotation. The plant was rotated about this axis at a constant 2 rpm for gravity compensation. At the same time a centrifugal force was imposed by rotation about the vertical axis of the compensator; this force was varied from $10^{-7} \times g$ to $3 \times g$ by varying the rotational speed about the verti-

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FIG. 192.—Influence of centrifugal force direction on the growth of roots and shoots of compensated *Avena* seedlings. The direction of the force, $0.1 \times g$ at the seed level, is indicated by the arrows. O, compensated; initial orientation of embryonic shoot centrifugal, embryonic root centripetal. I, compensated; initial orientation of shoot centripetal, root centrifugal. C, noncompensated, stationary; initial orientation of shoot "up," root "down."

FIG. 193.—Effect of various centrifugal forces on the elongation of compensated *Avena* shoots (A) and roots (B) when the initial orientation of the shoot apex was either centripetal or centrifugal.

cal axis. Plants were grown in complete darkness. Lengths and fresh weights of the organs were measured ca. 70 hours after planting.

PROGRESS REPORT

Figure 192 illustrates the alteration of growth patterns elicited in compensated plants by reorientation in a centrifugal field initially $10^{-1} \times g$. A force of this magnitude is clearly sufficient to produce a strong tropic reorientation—the shoots in sample O (initially oriented centrifugally) recurving in a negative geotropism to the direction opposite to that of the field, the roots

(initially oriented centripetally) recurving in a positive geotropism. In comparison with sample I, where the organs were oriented initially in directions 180 degrees to those in O, the growth of the shoot is decreased when the shoot apex points initially in the direction of the field, i.e., centrifugally. With the root, centripetal orientation resulted in a reduction in growth rate. These reactions are also reassuring as to the validity of the assumption underlying the present approach, namely that the *elongation* of the organs under centrifugal force is indicative of their responses to gravitational force. Hering⁽²⁾ found that when plants were

FIG. 194.—Effect of orientation in a centrifugal field on the growth of compensated *Avena* organs. The data represent differences in length, centripetal orientation minus centrifugal orientation.

maintained in an inverted position with respect to gravity (analogous to the compensated seedlings in sample O with respect to field direction), the grand period of growth ended prematurely. Similarly, Bremekamp⁽³⁾ reported that the growth rate of an inverted *Pharbitis* was markedly curtailed in comparison to the growth rate of a normally oriented plant.

The lengths of various organs of compensated plants, for example the shoot (Figure 193A) and the root (Figure 193B), were not significantly affected by orientation when the centrifugal force imposed was less than $10^{-3} \times g$. However, material changes in these organ lengths do arise between the centripetal and centrifugal initial orientation of the shoot apex when the force is greater than $10^{-3} \times g$. In Figure 194 are shown the length differences (centripetal minus centrifugal) of the coleoptile, mesocotyl, entire shoot, and the root obtained at the various forces. (The differences in fresh weights of these organs are qualitatively similar to the

length differences.) At forces greater than $10^{-3} \times g$, the relationships appear to be sigmoidal in form. They suggest no discrete force threshold for effective response—the curve apparently tapers to background variation in the region between 10^{-2} to $10^{-3} \times g$ for all the organs. One might term this the “threshold” range of perception under the described experimental conditions. There also appears to be a reduction in the effect of orientation on the mesocotyl and root lengths at the highest centrifugal forces used, i.e., an impairment of mesocotyl and root elongation in the 2 to $3 \times g$ range.

It is of interest that the geotropic curvature of the oat coleoptile is enhanced when the shoot apex is oriented centrifugally under centrifugation, compared to the response with centripetal orientation.⁽⁴⁾ Similarly, the coleoptile of an inverted seedling curvatures more than one normally oriented when equal geostimuli are applied.⁽⁵⁾ In part, these findings indicate that

tropic curvature is not related to growth per se of an organ, but to differences in the growth rates of its laterally adjacent tissues. These transverse inequalities are caused by the transverse force vector derived from either gravity or angular acceleration. Since the longitudinal force vector may influence the magnitude of the georesponse, we have begun to examine the geotropism of shoots along whose longitudinal axis various forces have been continuously imposed.

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ON THE PHOTOTROPISM OF GRAVITY-COMPENSATED AVENA SEEDLINGS*

Jane Shen-Miller and Solon A. Gordon

PURPOSE AND METHODS

We are investigating the changes occurring at morphological, physiological and biochemical levels of organization in plants subjected to continuous reorientation of field direction (cf. Reference 1). This study was designed to determine if gravity compensation, i.e., continuous nullification of the directional component of the gravity vector, alters the phototropic responsiveness of the plant. Previously,⁽²⁾ we reported that the phototropic response of compensated plants was less than that of stationary plants. In this report, we will show that the mechanical motion involved in compensation can itself impair phototropic sensitivity, and that compensation per se actually enhances phototropism when the reference is the tropism of plants grown under approximately the same motional vectors but without compensation.

Seeds of *Avena sativa* (Victory I, Svalöf) were hydrated prior to planting on agar (1.5%) medium. The medium was poured into a 150-ml Erlenmeyer flask whose top was cut off at ca. 3.5 cm from the base. This was done to facilitate phototropic exposure of the seedling without interference by the glass. Four seeds were planted to a flask, each at an angle of 45° to the agar surface, to obtain a coleoptile orientation normal to the agar surface. The top and the base of the flask were sealed with masking tape, and its neck stoppered by a cotton plug. Each assembly was placed in a single-axis clinostat designed to hold ten such flasks; the longitudinal axis of the seedling was oriented horizontally on the axis of rotation. The speed of rotation was 2

rpm. In a number of experiments, quartz sand (16% H₂O w/w) was substituted for agar as a growth medium.

For the first 24 hours after planting, the seeds were exposed to red light. The plants were then grown in complete darkness except that a green safe-light was used for experimental manipulation. Exposure to phototropic stimulation took place ca. 70 hours after planting. A tungsten filament lamp with a blue glass filter (Corning 5-59) was used as the light source. Radiant densities were varied by changing the voltage across the lamp with a constant exposure time of 15 seconds. One seedling from each flask was selected for photoexposure, the rest being discarded. Shadowgraphs of the coleoptiles were taken at 1- or 2-hour intervals following photostimulation; their curvatures were measured subsequently with a goniometer. Curvatures were analyzed by Duncan's⁽³⁾ multiple range test. Curves were fitted by the method of least squares.

There were three experimental treatments before and after photostimulation. These were: C, gravity compensation (horizontal axis of rotation); R, rotation (vertical axis of rotation, obtained by tilting the clinostat 90°); N, normal stationary position. In the subsequent discussion, where there are paired treatment letters, the first represents the treatment before phototropic exposure, the second the treatment after.

PROGRESS REPORT

The previous study⁽²⁾ employed C and N treatments before and after photoexposure in a factorial design. Exposures were in the "first-positive" range of phototropic response, and curvature was measured two hours

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FIG. 195.—Curvature responses of *Avena* coleoptiles irradiated unilaterally. C, compensated; R, rotated on a vertical axis; N, normal stationary. (A) Incident radiant density 9.4 Kergs·cm⁻², seedlings grown on agar. Responses differ significantly at 5% level (NC vs. CC) and at 1% level (NC vs. RC and CC vs. RC). (B) Incident energy 7.8 Kergs·cm⁻², seedlings grown on sand. Responses differ significantly at 5% level (CC vs. RC) and 1% level (NC vs. CC and NC vs. RC).

after stimulation. We showed that compensation after photoexposure enables the full development of the phototropism by counteracting the opposing geostimulus. Though compensation did not change the radiant

densities at which maximum response occurred, normal stationary seedlings curved more than did compensated seedlings after unilateral exposure to various radiant densities.

In the present series, rotation on a vertical axis was included as an additional variable to determine the contribution of clinostat motion. In addition, the seedlings were shadowgraphed periodically with a phototropically inactive light source to indicate the time course of curvature development in the various treatments. The curves in Figure 195A show the effect of the various gravity treatments on the development of phototropic curvature of agar-grown seedlings after unilateral exposure to an energy in the first-positive response region. The curves in the lower portion of the graph represent seedlings not compensated after photostimulation. In these, the response at two hours was maximum and then appeared to fluctuate. Those seedlings that were compensated after irradiation (upper curves) continue to increase in curvature, again illustrating the removal of the counter stimulus of gravity. These upper curves, in particular, show that the tropic curvatures developed by plants both compensated (CC) or rotated (RC) during growth are less than those of the normal stationary (NC). Hence, motion of the clinostat per se reduced the response. However, phototropism was enhanced by compensation (CC) over its control for motion, the rotated treatment (RC). Similar effect of gravity treatments were obtained when the seedlings were grown on a sand medium (Figure 195B).

Figure 195 indicates that the curvature increased at a constant rate in the first 3 to 4 hours and then decreased. Arisz⁽⁴⁾ similarly reported that phototropic curvature increased linearly up to ca. 5½ hours; the maximum response was reached 6 hours after irradiation. Bräuner and Hager,⁽⁵⁾ in their study of the geotropic response of *Helianthus* seedlings, concluded that compensation following geostimulation did not influence either the time at which the maximal curvature was attained or the magnitude of the maximum. However, it reduced the rate of subsequent "straightening-out" of the curved seedling; i.e., it only decreased the counter georesponse. With *Avena* seedlings, we find that compensation after irradiation increases not only the magnitude of the maximum but also the time required to reach the maximum (Figure 195A). We differ with the conclusion of Bräuner, *et al.*, that the effect of the second stimulus is not expressed until the response to the first has fully developed; the *Avena* seedlings apparently begin to respond geotropically soon as phototropic curvature begins (compare lower curves to upper curves, Figure 195A).

In another group of experiments, we have measured the effect of gravity treatment on the length of shoots

of agar-grown plants. The growth measurements were made beginning ca. 70 hours after planting. The normal stationary shoots were longer than those either rotated or compensated, and the compensated shoots were shorter than those rotated.

But curvature of an organ occurs as a result of *differences* in growth rate of laterally adjacent tissues, and is not quantitatively dependent on growth rate per se. The differences in growth rate are, however, classically associated with differences in the auxin contents of adjacent tissues. Since the phototropic response of the compensated plant is significantly different from both its rotated and stationary controls, we are asking whether these differences derive from: 1) changes in the hormonal imbalance induced by unilateral irradiation, changes derived from the gravity treatments (altered rates of auxin synthesis or of transport of the hormone could account for these changes); and/or 2) lateral differences in the reactivity of the tissues to auxin.

Two sets of preliminary experiments are of interest with respect to the above alternatives. First, we have applied apically C^{14} -labeled indoleacetic acid to the tips of coleoptiles subjected to gravity treatments. More of the labeled auxin is transported basipetally to subjacent tissues in compensated shoots than in their rotated controls. However, we do not know as yet whether this enhancement is caused by an effect of compensation on the transport capacity per se or on the ability of the tissue to absorb the hormone from the site of application.

Second, equilateral irradiation with first-positive radiant densities decreases the elongation of the *Avena* shoot of compensated, rotated and stationary seedlings. The reduction is greatest in the compensated plants. When indoleacetic acid is supplied to these irradiated seedlings, all of the plants overcome the light-induced inhibition of growth, with the compensated plants actually growing longer than the stationary or rotated. It may be inferred that auxin is limiting growth regardless of gravity treatment. When auxin availability is increased by exogenous supply, the compensated shoots appear to be more responsive to the hormone than plants grown without nullification of the directional component of the gravity vector.

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GEOTROPISM IN SIMULATED LOW-GRAVITY ENVIRONMENTS*

Richard R. Dedolph, Solon A. Gordon, and Donald A. Oemick

PURPOSE AND METHODS

Implicit in clinostat experiments is the concept that rotation about a horizontal axis cancels biologically the directional component of the gravity force vector. This concept assumes only that the angular velocity of rotation in the horizontal clinostat should be great enough to prevent a response to geostimulation during rotation, and yet not so great as to impart geotropically effective unidirectional centrifugal forces to the organism. One should be able, therefore, to impose vectorially, various defined gravity forces on plants during rotation by altering the angle of inclination of the rotational axis, and so be able to test the validity

of the nullification concept. In this fashion, we have determined the relationship between geotropic curvature response and the calculated degree of nullification of the directional component of the gravity force vector. Concomitantly, we have approximated the range of forces that are required for geotropic response in this type of geostimulation.

Geostimuli were imposed by tilting the rotation axes of clinostats rotating at 2 rpm, nullifying a portion of the directional component of the gravity force vector. The portion not nullified equals the sine of the angle of inclination of the rotational axis.

Precise angular adjustment was made with a spirit level, a straight edge, and a carpenter's framing square (Figure 196b). As diagrammed, the sine of the angle of inclination equals 10/50 or 0.20. Plants grown on a

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clinostat with this orientation and rotating at an appropriate rate would be subjected to 0.20 g . In this study we will deal solely with responses to "inverted" gravity forces, i.e., where the initial direction of the

FIG. 196.—Planting and treatment diagram

embryonic coleoptile tip was, if not horizontal ("zero" g), downward.

Hulled oat seeds (*Avena sativa* cv. Victory 1, Svalöf, Sweden) were soaked 2 hours in water at about 21°C. They were then planted in a 2-cm thick layer of moist quartz sand (about 15% H₂O by weight) in the bottom of 250-ml beakers. Orientation of the seeds is shown in Figure 196a. The beakers were placed on clinostats rotating at 2 rpm, with rotational axes tilted at various angles.

Seventy-two hours after the initial soaking, the sand was washed from the seedlings and the directional orientation of roots and coleoptiles was determined. From these data the percent germination and percentage of the population responding to the inverse gravitational force were calculated. The response noted was reversal of normal directional growth. When a coleoptile grew downward into the sand prior to emergence or when the tip of a coleoptile pointed toward the sand surface following emergence, it was considered a responding organ. Roots that had grown toward or penetrated the sand surface were likewise considered responding organs.

Mean percentages of responding individuals were plotted and approximated by least squares curves (Figure 197). The equations employed afforded a 95% reduction of the sum of squares of deviation about the general mean of all points in the 0.00 through 0.14 g range. Differences between germination percentages at the various g loads were tested by chi-square methods. Differences in the mean number of roots per plant from

FIG. 197.—Percent of roots and coleoptiles responding by inverted growth habit to varying gravity forces. □ roots; ○ coleoptiles

the various gravity treatments were tested by analysis of variance.

PROGRESS REPORT

The percent of both roots and tips responding shows a steady increase from 0.00 *g* to 0.08 *g* for coleoptiles and to 0.06 *g* for roots (Figure 197). Initially the rates of increase are high; as greater *g* forces are imposed, the rate diminishes. No discrete response threshold is evident (Figure 197). The percent of individuals responding diminishes uniformly.

When sand-grown plants were rotated during growth, with the rotational axis of the clinostat horizontal, a portion (ca. 20 to 30%) responded anomalously as if a geotropic stimulus were present. It may be considered possible that the small centrifugal force imparted by rotation elicited this anomalous response. However, the centrifugal force, with the 2 rpm rotation rate and a 1-cm displacement from the rotational axis, equals 4.5×10^{-5} *g*. If effective, this force should have induced the roots to grow away from the axis of rotation and the coleoptiles to grow toward it; such responses were not evident. Further, the curves (Figure 197) suggest if the individuals were responding

to this centrifugal force their number would be extremely small in contrast to that found. We, therefore, conclude that the centrifugal force exerted by rotation was too small to elicit a response of the magnitude observed when rotational axes were horizontal.

CONCLUSIONS

The percent germination (averaging 67%) and the number of roots per plant (averaging 4.2%) did not materially differ among the various gravity treatments.

The data indicate that the geotropic responses of roots and coleoptiles of sand-grown plants were not materially enhanced by forces greater than 0.06 to 0.08 *g*, respectively. There is no threshold *g* value, i.e., no value below which this directional growth response is not lessened.

This work demonstrates the alteration of effective geostimulus by nullification of the directional component of the gravity-force vector. It appears, therefore, that horizontal clinostat rotation at appropriate rates does nullify gravity as a determinant of directional growth of plants.

AUXIN TRANSPORT AND GEOELECTRIC POTENTIALS*

Shamshad M. Naqvi, Richard R. Dedolph, and Solon A. Gordon

PURPOSE AND METHODS

Asymmetry of indoleacetic acid distribution has been associated with the development of plant bioelectric potentials. In the absence of auxin, no detectable potential develops, but when auxin is supplied, auxin asymmetries caused by differences in transport give rise to bioelectric potentials.^(1, 2) Recently,⁽³⁾ it was demonstrated that under anoxia, where no potential develops, corn coleoptiles still showed geotropic curvature response. This raised three related questions: a) Are geoelectric potentials a mandatory consequence of auxin transport-induced asymmetries? b) If gravity-induced asymmetries occur under anoxia, do these differ from those established in air? c) Is the differential growth rate (curvature) associated with differing amounts of auxin transport on upper and lower sides of a geostimulated plant organ?

To answer these questions, the auxin transport in vertically and horizontally oriented coleoptile segments under aerobic and anaerobic conditions were studied.

This was done by excising 10-mm coleoptile sections

commencing 1 mm below the tip from dark grown corn plants with coleoptiles 2–3 cm in total length. These 10-mm tissue sections with leaf removed were then pressed base down onto a stainless steel razor blade protruding 2 mm above the surface of a 1.5% agar-gel block, $2.5 \times 35.0 \times 3.0$ mm, bringing the coleoptile section base into contact with the surface of the gel. Six sections were arranged along each blade so that the vascular bundles of each of the coleoptile sections were on opposite sides of, and equidistant from, the protruding blade. The blade was so placed in the agar block that it bisected the 3-mm dimension, forming 2 receiver blocks of equal size. A small "maintenance" block, $3.7 \times 4.0 \times 1$ mm, containing 2% sucrose and 0.2 mg/l IAA was then placed on, and covered, the apical cut surface of each coleoptile section. Vertical orientation of tissues was maintained during all stages of assembly. The temperature during manipulations and transport was 25°.

As they were prepared, assemblies were placed alternately into a chamber preflushed and continuously flushed with water-saturated nitrogen, or a chamber preflushed and continuously flushed with moist air. A

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3-hour equilibration period followed, during which the flow rate of the nitrogen into the gas chamber was sufficient to reduce the oxygen concentration by dilution to less than 0.01%. A positive gas pressure was maintained during experiments to minimize the effects of possible small leaks in the system.

After the equilibration, the maintenance blocks were replaced with similarly sized donor blocks containing 0.2 mg/l methylene-labeled C^{14} -IAA (specific activity 13.3 Ci/mole) and 2% sucrose. Following replacement of the maintenance blocks with donor blocks, 5 randomly selected assemblies in each gas environment were reoriented to a horizontal position and the remaining 5 assemblies in each chamber remained vertical. After 2 hours of transport, net activities of the donor blocks, tissues, and receiver blocks from each assembly were determined.

Net activities and the data derived from these net activities were summarized by analysis of variance. Locations of differences among the means were delineated by multiple range tests.⁽⁴⁾

PROGRESS REPORT

The C^{14} -IAA contents of donor blocks from assemblies under anaerobic conditions were higher and the contents of the tissue segments lower than C^{14} -IAA contents from assemblies under aerobic conditions (Table 96). No differences in donor-block or tissue activities ascribable to orientation to gravitational force was discerned. The average counts of receiver blocks from anaerobic assemblies showed roughly half of the activity of receivers from aerobic assemblies (234 cpm *vs.* 460 cpm). Horizontal orientation de-

creased the average activity of the two receiver blocks from vertically oriented aerobic assemblies. Under the anaerobic environment, no difference in average counts from the two receiver blocks from assemblies differing in orientation could be found.

Regardless of gaseous environment, the lower receiver blocks of horizontally oriented assemblies showed higher average counts than the upper receiver blocks from the same assembly. Under aerobic conditions the average counts of the lower receivers were about 2.6 times greater than those of the upper; under anoxia this ratio is 3.9. Right and left blocks from vertical assemblies in a single gaseous environment were not materially different.

These data show that the radioactivity in coleoptile segments is decreased by anoxia but is unaffected by orientation in either air or nitrogen. Anoxia reduces by half the auxin moved by coleoptile segments from donor to receiver. Horizontal orientation of the tissues decreases auxin transport into the receiver blocks in air but not under anoxia. The disparity in auxin content between the upper and lower receivers is, if anything, more marked under anoxia than in air.

In the compartmentalized transport system utilized, auxin must be absorbed from the donor block by tissues prior to transport through tissue to the receiver block. From the activity data (Table 96), one may determine the percentage distribution of absorbed C^{14} -IAA in the various parts of the assemblies. Of primary interest is the determination of what portion of the total absorbed C^{14} -IAA is transported.

When the activity data of Table 96 are re-expressed as percentages (Table 97), one finds that roughly twice

TABLE 96. THE EFFECT OF AEROBIC AND ANAEROBIC CONDITIONS ON THE BASIPETAL TRANSPORT OVER 2 HOURS OF C^{14} -IAA IN 10-MM CORN COLEOPTILE SECTIONS

The data presented are average counts per minute of 5 assemblies, each containing 6 coleoptile sections

Assembly parts	Treatments							
	Horizontal				Vertical			
	Aerobic		Anaerobic		Aerobic		Anaerobic	
Donor blocks	1670 _b *		2360 _a		1677 _b		2410 _a	
Tissues	785 _a		386 _b		826 _a		374 _b	
Receiver blocks	422 _b		229 _c		498 _a		238 _c	
By halves	Upper	Lower	Upper	Lower	Right	Left	Right	Left
	116 _a	306 _a	47 _c	182 _c	251 _b	247 _b	118 _a	120 _a
Ratio: Lower Upper	2.6 _a		3.9 _b					

* Means within a row not followed by the same subscript differ at odds greater than 99:1.

TABLE 97. THE PERCENTAGE DISTRIBUTION OF ABSORBED C¹⁴-IAA IN 10-MM CORN COLEOPTILE SECTIONS AND RECEIVER BLOCKS AFTER 2 HOURS OF AUXIN TRANSPORT

	Treatments							
	Horizontal				Vertical			
	Aerobic		Anaerobic		Aerobic		Anaerobic	
Percentage of applied								
In tissues	27.3 _a *		13.0 _b		27.5 _a		12.4 _b	
In receivers	14.7 _b		7.7 _c		16.6 _a		7.9 _c	
Total	42.0 _a		20.7 _b		44.1 _a		20.3 _b	
By halves	<u>Upper</u>	<u>Lower</u>	<u>Upper</u>	<u>Lower</u>	<u>Right</u>	<u>Left</u>	<u>Right</u>	<u>Left</u>
	4.0	10.7	1.6	6.1	8.4	8.2	3.9	4.0
Percentage of absorbed transported to receiver blocks	35.0 _a		37.2 _a		37.6 _a		38.9 _a	
By halves	<u>Upper</u>	<u>Lower</u>	<u>Upper</u>	<u>Lower</u>	<u>Right</u>	<u>Left</u>	<u>Right</u>	<u>Left</u>
	9.6 _d	25.4 _b	7.6 _d	29.6 _a	18.9 _c	18.7 _c	19.3 _c	19.6 _c
Ratio: $\frac{\text{Lower}}{\text{Upper}}$	2.6 _b		3.9 _a					

* Means within a row not followed by the same subscript differ at odds greater than 99:1.

as great a fraction of the applied auxin in aerobic tissues or receivers as in anaerobic. Of the auxin absorbed, roughly one-third is transported regardless of atmosphere or gravitational orientation. Of the auxin absorbed by horizontally oriented tissues, a slightly greater portion is transported to the lower half of the receiver under anoxia than to its counterpart in air. The almost identical ratios of the percentages of absorbed auxin in upper and lower blocks in air and nitrogen compared to corresponding ratios of counts (Table 96) attests to the strict proportionality of auxin transported to that absorbed. These percentage data collectively show that atmospheric environment greatly influences the amount of applied auxin that is absorbed. Atmospheric environment, however, little influences the asymmetry in the amounts of auxin transported in horizontally oriented tissues or the fraction of that absorbed which is transported.

From these experimental results, it is concluded that the development of geoelectric potential is not a mandatory consequence of asymmetric auxin distribution. Geoelectric potentials are not evident under anoxia where asymmetric auxin distribution does occur. Gravity-induced auxin asymmetries under anoxia are similar to those in air, but greater in magnitude. Anoxia affects the net absorption of auxin, but little

influences the fraction of absorbed auxin which is transported.

CONCLUSIONS

Corn (*Zea mays* L.) coleoptile segments readily transport indoleacetic acid under anaerobic conditions as well as aerobic conditions. Gravity-induced asymmetry of transport of indoleacetic acid in horizontal segments is greater under anaerobic conditions than under aerobic conditions. As geoelectric potentials do not occur under anaerobic conditions, it is concluded that geoelectric potentials are not a mandatory consequence of asymmetric auxin distribution. Anaerobic atmospheres roughly halved auxin absorption but had little effect on the transport of auxin once it was absorbed.

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THE ROLE OF AUXIN IN THE GEOTROPISM OF COMPENSATED AVENA SEEDLINGS*

Richard R. Dedolph, Shamshad M. Naqvi,† and Solon A. Gordon

PURPOSE AND METHODS

Geotropism as well as other geophenomena is interpreted as gravity-induced asymmetry in auxin concentrations. We find an enhanced response to geostimulation of plants grown in clinostats where the directional component of the gravity vector is continuously nullified by rotation on a horizontal axis (compensation).⁽¹⁾ This study was undertaken to assess the causal role of auxin in this enhanced georeponse. We will show that differences in reactivity to auxin rather than changes in auxin availability are largely responsible.

Oat seeds were planted near the center and just below the surface of a 2-cm thick layer of moist quartz sand. During the 72-hour growth period seedlings were subjected to one of the three gravity conditions depicted in Figure 198.

Radioactive Auxin Transport

Beginning 1 mm below the tip, a 7.0-mm coleoptile section was excised from coleoptiles grown under each of the gravity treatments (Figure 198). After removal of the primary leaf, this cylindrical tissue section was placed base down around a nylon bristle rising vertically from the center of a square Plexiglass plate on which a block of agar had been previously centered. For acropetal transport, this agar block served as a "donor" and contained 0.4 mg/liter methylene C¹⁴-labeled IAA. A similar "receiver" block of agar was placed on the acropetal end of the tissue segment. For basipetal transport, the positions of the donor and receiver blocks were reversed. The entire assembly was attached with putty to a beaker (Figure 199), which was then returned to the appropriate gravity treatment. The time of assembly was less than one minute; consequently, the effects of unidirectional exposure to gravitational stimulation were minimized.

After 2.5 hours, the donor blocks, tissues, and receiver blocks from four identically treated assemblies were pooled according to part. Their radioactivities were determined by scintillation counting.⁽²⁾

Factorial combinations of pre- and postassembly treatments of horizontal rotation and stationary controls (Figure 198) were employed. Vertical rotation in the pre- and postassembly period was used to afford an

experimental index of the effect of rotation per se. Five additional sets of percentage ratios were generated from these counts-per-minute data. The effect of rotation per se was tested in each of these eight data sets by analyses of variance, comparing the data from samples vertically rotated in the pre- and postassembly conditions with those held in the vertical stationary conditions during these periods. No material differences in auxin transport attributable to rotation or rotation interactions were discerned, validating biologically the variance analysis utilizing the factorial arrangement of treatments in the pre- and postassembly periods.

Endogenous Auxin

Six 5.0-mm coleoptile tips were excised from plants grown under the different gravity conditions and placed, base downward, upon an agar block resting on a glass plate. The plate with agar block and tips was then attached to a beaker with putty (Figure 199) and returned to the gravity condition under which they

a. PLANTING DIAGRAM b. TREATMENT DIAGRAM

FIG. 198.—Planting and treatment diagram

* Work supported in part by the National Aeronautics and Space Administration.

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were grown. Assembly time was always limited to less than one minute. After two hours, tips were removed and the blocks were allowed to equilibrate. These large blocks were then divided into blocklets for bioassay by the *Avena* curvature test.

Tissue Reactivity to Auxin

Coleoptiles were grown under compensation, with vertical stationary controls, for 72 hours after they were decapitated (2 mm), grown for 2 more hours under the same gravity conditions and again decapitated (2-3 mm). After slightly raising the primary leaf, an agar block containing 100 $\mu\text{g/liter}$ IAA was placed unilaterally on the stump. The coleoptiles were then kept in the vertical stationary condition for ninety minutes, shadowgraphed, and the curvatures recorded.

PROGRESS REPORT

Less activity and a lower percentage of the auxin applied were found in the tissues and receiver blocks under acropetal than under basipetal transport conditions. Acropetal auxin application decreased the percentage of applied activity that was absorbed by about threefold (29 vs. 9%), but increased tissue retention of the auxin (81 vs. 61%).

Acropetal transport of absorbed basally applied auxin was about half that of the absorbed apically applied auxin (19 vs. 39%). These data show that there is relatively little absorption of basally-applied auxin, but once absorbed, net acropetal transport of auxin is about half as great as basipetal. These findings support longstanding evidence of acropetal transport in *Avena* coleoptiles.^(3, 4)

FIG. 199.—Diagram of assemblies used as auxin transport and production studies.

TABLE 98. EFFECTS OF PREASSEMBLY GRAVITY TREATMENT WITH ACROPETAL AND BASIPETAL AUXIN APPLICATION ON COUNTS PER MINUTE IN TISSUES AND RECEIVERS AND ON PERCENT OF APPLIED AUXIN IN RECEIVER BLOCKS AND IN TISSUES PLUS RECEIVER BLOCKS

Preassembly condition	Data showing material difference							
	Cpm in				% of applied auxin in			
	Receiver blocks		Tissue sections + receiver blocks		Receiver blocks		Tissue sections + receiver blocks	
	Acro-petal	Basi-petal	Acro-petal	Basi-petal	Acro-petal	Basi-petal	Acro-petal	Basi-petal
Vertical stationary	38	175	170	460	2.1	10.3	9.5	27.1
Horizontally rotated	26	198	156	502	1.5	12.0	9.3	30.2
	LSD* ₀₅ = 13 cpm		LSD ₀₅ = 27 cpm		LSD ₀₅ = 0.9%		LSD ₀₅ = 2.1%	

* Least significant difference.

TABLE 99. THE EFFECT OF GRAVITY TREATMENTS DURING GROWTH TESTING PERIOD ON THE CURVATURE RESPONSE TO UNILATERALLY APPLIED CURVES

Growth condition	Test condition	Curvature in degrees
Vertical stationary	Vertical stationary	19.6*
Horizontal rotated	Vertical stationary	22.0*

* These two values differ significantly from each other at odds >19:1.

Direct comparison of the counts-per-minute data in receivers following acropetal and basipetal transport suggests that transport in the acropetal direction is only one-sixth of that in the basipetal. The more rigorous percentage comparisons that include absorption as a contributive factor indicate that a reduction by a factor of one-third is attributable to a decreased net absorption, and reduction by a factor of only one-half to a decreased net transport. Since auxin absorption in corn coleoptiles⁽⁵⁾ and pine shoots⁽⁶⁾ is correlated with metabolic activity and auxin transport is not, perhaps the reputed strict basipolarity of auxin transport⁽⁷⁾ is a reflection of metabolically induced differences in absorptive efficiency between the upper and lower ends of the tissue segment. Comparisons of various tissue segment lengths, indicating a decrease of transport polarity with shorter tissue segments,^(4, 7) support this inference.

When the polarity of transport and gravity treatments are jointly considered, the pronounced influence

of the pre- but not postgravity treatment becomes apparent. The counts per minute of receiver blocks, tissue sections plus receiver blocks, percentage of applied auxin in receiver blocks, and percentage of applied auxin in tissue sections plus receiver blocks, all are influenced by pre- but not postassembly gravity treatment (Table 98). In each of these data sets, the enhancement effect of horizontal rotation was elicited only in the assemblies testing basipetal transport. No difference attributable to gravity treatment was apparent in the true transport index, the percent of absorbed auxin found in receiver blocks.

From these data we conclude that auxin transport is not modified by rotation on either a horizontal axis (compensation) or a vertical axis. However, auxin uptake by the tissues, a metabolically dependent component of the transport system, is consistently increased by compensation. It may, therefore, be suggested that metabolism is accelerated by nullification of the directional component of the gravity-force vector. It may also be suggested that the increased curvature response to geostimulation of horizontally rotated coleoptiles⁽¹⁾ results from enhanced metabolism and not an increase in auxin asymmetry based on changes in transport rate.

Auxin production plus transport from *Avena* tips was determined. The tips were cut from seedlings that were compensated, vertically rotated, and vertically stationary. Bioassay of the receiver blocks yielded respective mean curvatures of 3.5, 4.3 and 2.5 degrees. These means do not differ significantly ($P = 0.05$). If we assume that the amount of auxin diffusing from the excised tip is an index of the rate of auxin production immediately prior to excision, we may infer that the horizontal rotation did not enhance the geotropic response of the plant by altering the auxin supply.

The above inference does not exclude the possibility that an increased metabolic activity induced by horizontal clinostat rotation might not also cause an increased auxin production in *intact* organs.

An enhancement of curvature response to unilaterally applied auxin results from compensation (Table 99). This increase in reactivity is also suggestive of a metabolic enhancement in the seedling by previous compensation.

Collectively, these experiments indicate that enhanced plant responses to horizontal clinostat rotation, where rates of rotation are sufficient to nullify the directional component of the gravity-force vector, are caused primarily by increases in metabolism and not to modification of auxin availability. These data do not support hypotheses recently advanced elsewhere that the polarity of auxin transport is based on gravitational sedimentation of cell inclusions.

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ENVIRONMENTAL RADIATION STUDIES: STATEMENT OF SCOPE AND COMMITMENT

Philip F. Gustafson

The initial purpose, and one of continuing major emphasis, was the investigation of the levels of fission-product and natural radioactivity present in the Chicago environment and the dose to the population arising from it. This involves a study of radioactivity in air, precipitation, soil, and vegetation at Argonne, as well as in foodstuffs and biological samples collected in the Chicago area. Primary reliance is placed upon low background gamma-ray spectrometry for the determination of the composition and the concentration of radioactivity in these samples, with growing dependence upon radiochemical techniques where this is necessary. Usually these measurements have utilized conventional electronic equipment; although to solve special problems it has been necessary to design and construct new devices or modify existing ones. An example in this latter category is the GASP, a balloon-borne gamma-ray spectrometer system, used to measure the distribution and composition of high altitude nuclear debris as part of the Balloon-Borne Gamma-Ray Spectroscopy Program. The spectral data from both the environmental measurements and the balloon program are processed almost exclusively by electronic computers.

Investigations of the temporal variation of both fission-product (caused by radioactive decay, changes in deposition rate, and ground dispersal) and natural radioactivity (caused by differences in radon retention in soil due to moisture content and snow cover) are necessary to evaluate human radiation exposure from external sources properly. The relationship between the levels of radioactivity present in foodstuffs (made as part of the Tri-City Diet Sampling Program) and those in man is being investigated. This relationship provides a clearer understanding of radionuclide metabolism and permits more reliable estimates of internal radiation exposure to be made. The scope of these studies includes meteorology, geology, soil chemistry, and other scientific disciplines pertinent to the investigation of the problem. Because of the limitation in staff, it is not possible to pursue all aspects of these studies with equal determination. The emphasis

changes as the relative importance of the radiological parameters change. For example, open-field dose determinations are important during and shortly after nuclear testing, while currently, more emphasis is placed upon the behavior of long-lived fission radionuclides. Because many of the facets of environmental radiation under consideration in this program are not restricted to one locality, the results of these investigations may be used with confidence on a broad geographic and temporal scale.

A field trip was made by one of us (SSB) to California in October, 1964 as part of the continuing study of environmental radiation conducted in cooperation with HASL, LRL, and Rice University. Comparisons of field instrumentation (ionization chambers and scintillation spectrometers) were made under conditions of low cosmic-ray dose rate (deep in a mine shaft) low terrestrial dose rate (open-pit asbestos mine) and high terrestrial dose rate (over bare granite outcrop). The field studies have led to a more precise measurement of total environmental dose rate as well as the dose rate due to individual components (cosmic radiation, uranium, thorium, potassium), and have established more uniform calibration and standardization procedures.

The results of environmental sampling in the Chicago area during 1964-65 will be presented in the following reports. In particular, the relationship between dietary levels of Cs^{137} and those found in man will be discussed in some detail. The methods used in processing spectral data will be presented along with the radiochemical procedures and instrumentation unique to the determination of trace elements not measurable by other means. The high levels of Cs^{137} present in fresh-water fish, observed during the course of the food study, has led to the investigation of Cs^{137} in the fresh-water ecosystem. Results of this new study made during the summer of 1965 will be given. Development of a new spectrometer system, GASP-II, to be used in the balloon program will be discussed, as well as other recent aspects of the program. Performance data for the two new low-background shields will also be given.

GAMMA-RAY SPECTROMETRIC MEASUREMENTS OF ENVIRONMENTAL SAMPLES

Philip F. Gustafson, Sarmukh S. Brar, Stanley E. Muniak, and Donald M. Nelson

PURPOSE AND METHODS

These investigations were the initial ones made into man's radiation environment, and they have provided continuity to our effort. The methods used are those of conventional low-level gamma-ray spectrometry and remain essentially unaltered from those discussed in the previous annual report.⁽¹⁾

Periodic measurements of radioactivity in the external environment (air, precipitation, and soil measurements) permit an evaluation of the magnitude and composition (i.e., specific radionuclides) giving rise to the external radiation exposure as a function of time. Similarly, investigations of foodstuffs (Cs^{137} in the Chicago diet) make it possible to assess the internal dose contribution from specific radioelements. The fission-product radioactivities in these various media, including man, are closely interrelated. By examining them concurrently at one site, it is possible to define more clearly these relationships (see section on Relation between Cs^{137} in the Diet and in Man). The results of such close inspection may be used as a basis for predicting the future course of fission-product behavior. In a broader sense, these investigations yield fundamental information regarding the atmosphere and the biosphere and their relation to man that may not be obtained effectively in any other way.

PROGRESS REPORT

Air, Precipitation, and Soil Measurements

The spring maximum in the concentration of fission products in surface air has been followed by means of Cs^{137} measurements at Argonne for a number of years. The highest concentration observed at Argonne came during June and July of 1963, and amounted to ~ 0.170 pCi Cs^{137} /SCM (standard cubic meter) of air. In 1964 the maximum occurred at approximately the same time, but was of lower magnitude, amounting to ~ 0.110 pCi/SCM. A further decrease in concentration was noted in 1965, ~ 0.045 pCi/SCM, which occurred in May and June. There exists a close correlation between the relative amplitudes of the spring maxima and the contents of the atmospheric reservoir (specifically the inventory of Sr^{90} in the stratosphere). The reason for a change in the time of occurrence of the spring maximum are not understood; however, there appears to be a cyclic behavior which has been observed over much of the northern hemisphere.

There is also a close relation between the magnitude of the spring maximum in surface air and the amount

of Cs^{137} deposited during that particular year; this relationship is influenced by the amount of rainfall during that year. Deposition is a function of the concentration of air-borne radioactivity and the amount of precipitation removing this activity from the air and depositing it on the ground. The deposition of Cs^{137} at Argonne was also greatest in 1963, with ~ 78 mCi/mi² deposited. In 1964 ~ 42 mCi/mi² were deposited, and up to July 1965 another 15 mCi/mi² had fallen, making a total accumulation on the ground at Argonne of 330 ± 20 mCi/mi².

By the autumn of 1964 the total accumulation on the ground of all of the important gamma-emitting fission nuclides, with the exception of Cs^{137} , was decreasing because losses by radioactive decay exceeded new deposition. Even in the case of Cs^{137} new deposition barely overcame the loss by radioactive decay. The open field (unshielded) exposure dose rate due to fission products has actually been decreasing since mid-1963, primarily because of the decay of Zr^{95} - Nb^{95} (65-day half-life) which is the principal source of dose rate at early times. The dose rate from fission products was 1.7 $\mu\text{R}/\text{hour}$ in June 1965, compared with 2.2 $\mu\text{R}/\text{hour}$ a year earlier, and a high value of 7.5 $\mu\text{R}/\text{hour}$ in June 1963. By June 1965 approximately 60 percent of the total fission-product dose rate came from Cs^{137} alone. The annual open field exposure from fission products was 19 mR/year in 1964, compared with 35 mR/year in 1963; and the first six months of 1965 have seen a dose contribution of approximately 8 mR. The annual external dose from natural radioactivity and cosmic rays at Argonne is 108 mR/year. Furthermore, a shielding factor of 2 must be applied to the open field dose from fission products to take into account the shielding properties of buildings, houses, and the like. Hence the dose contribution from fission products increased the exposure to man by nearly 9 percent in 1964, and presumably the increment will be somewhat smaller during 1965.

There were two small inputs of new fission products into the atmosphere during 1964-65, both from Chinese nuclear tests; one on October 16, 1964, the other on May 14, 1965. Both led to increases in air-borne radioactivity, primarily Ba^{140} - La^{140} and the other short-lived nuclides. Ground deposition at Argonne of radioactivity from these tests was minimal.

Cs^{137} in the Chicago Diet

The concentration of Cs^{137} in various foodstuffs purchased in the Chicago area indicated decreasing trends

FIG. 200.—Daily Cs^{137} intake (pCi/day) derived from five food categories FIG. 201.— Cs^{137}/K ratio in Chicago residents and Chicago diet

from 1964 into 1965, in accord with the belief that ^{137}Cs contamination of the food supply varies with deposition rate rather than with the total accumulation. This is illustrated in Figure 200 where the Cs^{137} con-

centrations in milk, meat, grain products, fruit, and vegetables are given in terms of daily intake (pCi/day). Consistently, the bulk of the daily Cs^{137} intake comes from milk, meat, and grain products, with the

FIG. 202.—Comparison of human and modified dietary Cs^{137}/K ratios

relative importance of these dietary sources varying with time. At present, the Cs^{137} level in milk appears to be dropping more rapidly than in either meat or grain products. The more gradual decrease in the Cs^{137} level in both grain products and meat is attributed to the fact that grain currently being marketed (and consumed) was actually harvested in 1963 and 1964. Thus grain products eaten by man reflect contamination levels of one or two years ago. Grain is also an important livestock feed, and thus meat contains higher concentrations of Cs^{137} than might otherwise be expected. This is a lag imposed by marketing practices rather than by biological processes such as uptake from soil and retention in the grain.

The total daily intake of Cs^{137} in the Chicago diet was greatest in January 1964, reaching a value of 240 pCi/day, and has decreased rather steadily since that time, attaining a value of 120 pCi/day in July 1965. In Figure 200 the extrapolated daily intake values are indicated based upon extensions of the trends observed through July 1965. These values form the basis for the prediction of future intake levels.

Relation Between Cs^{137} in the Diet and in Man

Comparisons between the diet levels of Cs^{137} and those observed in man by whole-body counting⁽²⁾ have been conducted using the concentration of Cs^{137} and the ratio of Cs^{137} to potassium, i.e., the $\text{Cs}^{137}:\text{K}$ ratio. This

ratio, in the diet and in man, is shown as a function of time in Figure 201. Ignoring the rather abrupt variations in diet ratio during 1961 and 1962, there is a similarity between the ratio in the two media. There is a displacement in amplitude, and the maximum value of the ratio in the diet is reached about 4 months prior to its appearance in man. The early variations in the diet ratio are attributed to the appearance of recent Cs^{137} in portions of the diet; Cs^{137} is not homogeneous throughout a given diet component, nor is it constant in relation to time. Because of the roughly 100-day biological half-life of Cs^{137} in man, such variations are not expected to be duplicated in the human whole-body ratio. Furthermore, because the contamination is not uniform, there is no reason to suppose that the concentrations of Cs^{137} measured in the diet study are the same as those in the diet of the people being measured by whole-body counting. A transformation was employed in an attempt to match the diet ratio curve to that of the ratio in man. The diet ratio was multiplied by a factor of 3, and delayed by 4 months so that the maxima coincide. The results of this transformation are shown in Figure 202. While the fit is quite good, a plausible explanation is needed. Pendleton Mays,⁽³⁾ at Utah, have postulated that the $\text{Cs}^{137}:\text{K}$ ratio will increase from one trophic level to the next higher level by a factor which is the ratio of the biological half-life of Cs^{137} and potassium in the animal

in question. The factor of 3 employed in making the transformation is within the spread of experimental values for the cesium and potassium half-lives in man. The extrapolated diet levels shown in Figure 200 have been used to obtain a total diet concentration and $\text{Cs}^{137}:\text{K}$ ratios for future levels. These are illustrated in Figures 201 and 202, and provide a basis for predicting future levels of Cs^{137} in man in the Chicago area. The effective half-life of Cs^{137} in man at present (effective in the sense that it is the result of continuous intake that varies as shown in Figures 200-202) is $2\frac{1}{2}$ years.

CONCLUSIONS

The concentrations of Cs^{137} in surface air during 1965 decreased to approximately 40 percent of the level observed in 1964. Similar decreases in the deposition of Cs^{137} were noted, and the total accumulation of Cs^{137} on the ground at the Argonne site amounted to $330 \pm 20 \text{ mCi/mi}^2$ by July 1965. The open field exposure dose rate due to fission products fell to 75 percent ($1.7 \mu\text{R}/\text{hour}$ in June 1965) of the magnitude observed during comparable periods in 1964.

The levels of Cs^{137} in foods have decreased in accord

with the theory that food contamination by this radionuclide is governed primarily by fallout rate rather than by the total accumulation on the ground. The relation between the $\text{Cs}^{137}:\text{K}$ ratio in the diet and that observed in man was examined with the following results: 1) the increase in ratio (by a factor of 3) is in accord with the ratio of the half-lives of Cs^{137} and potassium in man, and 2) the agreement between direct observation of the ratio in man and that derived from the diet provide a firm basis for the prediction of Cs^{137} body burdens in man over the next year or so.

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INVESTIGATION OF Cs^{137} IN FRESHWATER ECOSYSTEMS

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PURPOSE AND METHODS

The purpose of this study is the investigation of the dynamic processes in the freshwater system that lead to relatively high concentrations of Cs^{137} in fish. The initial interest in these fish stemmed from their importance as a source of food for man, and the possibility that they may be a source of high Cs^{137} intake in his diet.

The presence of high levels of Cs^{137} in fish became apparent in the course of the Tri-City Diet Study (in which Cs^{137} determinations of the Chicago portion of the study were made at Argonne). Originally no effort was made to select particular species of fish; ocean and freshwater fish were purchased in varying quantities from one sampling to another. Occasionally, high concentrations of Cs^{137} were observed that erroneously were attributed to ocean fish. A thorough examination of a number of ocean fish indicated Cs^{137} levels the range of 10 to 100 pCi/kg wet weight. Freshwater fish showed universally higher levels, in the

range of from a few hundred to several thousand pCi/kg. All fish examined were purchased in local food stores, and no attempt was made to obtain fish from remote localities. Measurements were also made of the potassium content (by means of K^{40}) of the same fish, and the ratio of Cs^{137} to potassium ($\text{Cs}^{137}:\text{K}$ ratio) was examined. The potassium content of marine and freshwater fish is similar. It is the $\text{Cs}^{137}:\text{K}$ ratio that is considerably greater in freshwater fish. This difference is probably due to the higher concentration of potassium in ocean water (380 ppm) than in freshwater lakes and streams (few ppm K). With an equal concentration of Cs^{137} in ocean and fresh water, the initial $\text{Cs}^{137}:\text{K}$ ratio would still be essentially 100-fold greater in fresh water. There is, furthermore, the likelihood of greater dilution of Cs^{137} in the oceans than in bodies of fresh water.

Because the levels of Cs^{137} in freshwater fish were on the order of 1000 pCi/kg, the possibility existed to study not only the distribution of Cs^{137} within a fish population, but throughout much of the freshwater ecosystem. These investigations can be carried out under natural conditions which are impossible to dupli-

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cate in the laboratory. The results of such investigations will, it is hoped, be useful in the study of the more general problem of environmental contamination of lakes and streams by a variety of pollutants.

PROGRESS REPORT

Initial inquiries were made of the Chicago Office of the U. S. Bureau of Commercial Fisheries to determine the quantity and origin of freshwater fish marketed in Chicago. In addition, this office supplied figures for the total fish production in the United States, fish imports, and an itemization of both marine and freshwater species. These figures showed that freshwater fish comprised about five percent of the total fish consumed by man in the United States. About 90 percent of the freshwater fish consumed are imported from Canada. The first observation permits freshwater fish to be placed in proper perspective in dietary importance on a nation-wide basis, and the second point suggests the importance of examining fish from outside the borders of the United States. Individuals, however, may eat substantial quantities of freshwater fish due to proximity to an abundant source of freshwater fish as well as to a variety of other diet-influencing factors.

Three research efforts were made during the summer of 1965 related to Cs^{137} in freshwater systems: 1) examination of whitefish from a number of different lakes, 2) examination of the major fish species from a specific lake, and 3) examination of urine specimens from individuals living in a remote area with an abundance of freshwater fish as part of their environment. Although it was anticipated that collection of fish would have to be done by the staff, the amount of cooperation from other groups directly involved in fish management and inspection made such fishing trips both unnecessary and impractical.

Canadian Sources

The Food and Drug Administration inspect, in their Chicago Office, whitefish coming into the U. S. market from a number of Canadian lakes. Permission was obtained to examine samples of this species after FDA inspection. This provided an abundant supply of whitefish from Canada, collected over a wide geographic area (from the Northwest Territories to southern Manitoba). The potassium contents of all the whitefish examined were similar. The Cs^{137} concentration, however, varied considerably (~ 160 pCi/kg in Great Slave Lake, Northwest Territories to nearly 2000 pCi/kg in fish from Mirond Lake, Manitoba) over the region studied. To a first approximation, the differences are related to the total deposition of Cs^{137} over the regions involved. There also appear to be differences due to the size of lakes involved (dilution

effects) and their drainage areas. The potassium concentration in most of these lakes is not available, and the effect of potassium on the $\text{Cs}^{137}:\text{K}$ ratio cannot be determined.

The Red Lake, Minnesota Study

A large number of perch (77 in number) from the same catch and of the same approximate size were obtained from Red Lake, Minnesota through Rupert Fisheries in Chicago. These were analyzed individually for both Cs^{137} and potassium by gamma-ray spectrometry. The distribution of potassium appeared to be a normal distribution, whereas that of Cs^{137} was log-normal, which finding is similar to observations of K and Cs^{137} in human populations. The average Cs^{137} concentration was 752 pCi/kg wet weight, and the average K content was 2.40 g/kg. Some 90 northern pike were also obtained via the same route from Red Lake and similarly examined. The average Cs^{137} concentration was 3621 pCi/kg and the potassium content was 3.33 g/kg. The pike of Red Lake feed largely upon perch, and these constitute two adjacent trophic levels in the teleost food chain. The $\text{Cs}^{137}:\text{K}$ ratio in perch was 313 pCi/g while the corresponding ratio in pike was 1087 pCi/g. The increase in ratio, up one level on the trophic scale, was 3.47 ($1087 \div 313$). This observation is in accord with the investigations of Pendleton and Mays⁽¹⁾ in reference to increases in $\text{Cs}^{137}:\text{K}$ ratio ascending the trophic scale. These investigators attribute the increase to the fact that the biological half-time of Cs^{137} is approximately 3 times longer than the biological half-time of potassium in the same animal. This enrichment of Cs^{137} relative to potassium in going up the food chain is in direct opposition to the behavior of Sr^{90} relative to calcium under similar circumstances. This results in not only an increase in the $\text{Cs}^{137}:\text{K}$ ratio, but a distinct increase in the absolute concentration of Cs^{137} .

Red Lake has proved to be an ideal location for making a detailed ecological study. It is situated, for the most part, on an Indian Reservation; the Indians have fishing rights, and market exclusively through Rupert Fisheries in Chicago, through a local fishery located at Redby, Minnesota (Red Lake Fishery Association). The lake has been studied by conservationists and limnologists for a number of years—especially by Professor Lloyd Smith of the University of Minnesota who has maintained a strong interest in the lake since 1949. There is also an Indian Hospital at Red Lake, maintained by the U. S. Public Health Service. Through the kind cooperation of the Red Lake Fish and Professor Smith, samples of the major fish species were obtained in addition to preserved specimens from past years. Lake water and bottom samples have also

been sent to Argonne by Smith's group. The lake water contains approximately 7 ppm potassium, and the analysis of 7 liters of water gave an upper limit for Cs^{137} of 1 pCi/liter, thus yielding a maximum $\text{Cs}^{137}:\text{K}$ ratio of ~ 140 pCi/g in the water itself. The Cs^{137} concentrations and the values of the $\text{Cs}^{137}:\text{K}$ ratio in the various species of fish were in accord with their feeding habits and position on the trophic scale. As indicated by the analysis of fish from past years, the Cs^{137} concentration is increasing with time, which suggests that the accumulation of this radionuclide in the lake remains in biologically available form. The few bottom samples, analyzed thus far, show higher Cs^{137} concentrations near the mouth of Blackduck River, the principal river emptying into the lake, with decreasing concentrations as one moves out into the lake proper.

The final portion of this study was made possible through the assistance of Dr. Bernie Orr, director of the Red Lake Indian Hospital, who supervised the collection of urine specimens from a number of local Indians. Analyses indicate that there is a correlation between urinary excretion of Cs^{137} and the consumption of fish; those who ate fish had higher Cs^{137} levels in urine. However, none of the subjects thus far examined can be called fish eaters. Many showed a distinct aversion to fish, and most of their protein came from beef, pork, and chicken. On the whole, the Cs^{137} -content of the Indian urine samples approximated that observed in Chicago residents. The study is being continued.

These preliminary studies during the summer of 1965 have aroused considerable interest in, and subsequent cooperation from a number of agencies connected totally or in part with freshwater fish. In addition, interest is now manifest in Canada through the Radiation Protection Division in Ottawa.

CONCLUSIONS

The inherently higher concentrations of Cs^{137} observed in freshwater fish, as opposed to marine species, is attributed to the lower $\text{Cs}^{137}:\text{K}$ ratio in ocean wa-

ters, and to the vastly greater dilution of Cs^{137} in the oceans.

Studies of whitefish from a number of Canadian lakes, spread over a large region, showed variations in Cs^{137} concentration related to Cs^{137} deposition over the regions in question. Of somewhat lesser importance are the effects of dilution and drainage area of the lakes involved.

The variation of Cs^{137} in fish caught in Red Lake, Minnesota is in accord with the feeding habits and position of the fish on the trophic scale. Of particular interest is the increase in $\text{Cs}^{137}:\text{K}$ ratio by a factor of 3.5 in going from perch to pike (prey to predator). Analyses of fish caught prior to 1965 in Red Lake show that the levels of Cs^{137} in fish have increased with time, indicating a dependence upon total accumulation of Cs^{137} in the ecosystem, and its continuing availability. Analyses of bottom samples from Red Lake show promise of describing the Cs^{137} deposition contours in the lake due to water currents.

Measurements of Cs^{137} in urine samples from the Indian population surrounding Red Lake indicated higher levels in fish eaters. However, the study must be considered only a partial success thus far, as it has failed to prove the supposition that persons eating substantial quantities of freshwater fish will have correspondingly high Cs^{137} body burdens.

Red Lake, in all aspects considered thus far, appears to be a most promising site for the continued study of Cs^{137} in a freshwater environment. Cooperation with both fisheries and U. S. Public Health Service personnel has been most fruitful and will continue. A more detailed study of Red Lake and its surrounding population is planned for the summer of 1966.

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BALLOON-BORNE GAMMA-RAY SPECTROSCOPY PROGRAM

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PURPOSE AND METHODS

The purpose of this program is to locate radioactive nuclear test debris in the upper atmosphere by means of a balloon-borne scintillation detector system. Two systems have been designed, built, and are now operational. One, the GASP system, designed and built by

the Electronics Division at Argonne measures and telemeters a spectrum of the surrounding gamma radiation to a ground-based station. The circuitry and operating characteristics of this device were discussed in the previous annual report.⁽¹⁾

A second system, designated the GIGI, was designed and constructed by the AEC Health and Safety Laboratory in New York. The detector consists of a cylin-

* Electronics Division

dricial NaI(Tl) crystal 1 in. in diameter and 1¼ in. long which responds to both gamma rays and charged particles that lose more than 0.3 MeV energy within the medium. Other than having a low energy threshold, the GIGI has no spectral capability. Count-rate information is telemetered, and ground detection is accomplished in a fashion identical to that used with the GASP.

Experimental flights have been made at both San Angelo, Texas (Goodfellow Air Force Base) and Rantoul, Illinois (Chanute Air Force Base) to determine the detection sensitivity, stability, and temperature response of both the GASP and GIGI circuits. In addition, the over-all usefulness of the phoswich concept has been examined. These flights using the GASP indicated reasonable gain stability over the range of temperatures normally encountered, which could be improved even further by continuous calibration during flight using a Na²² source accompanying the device. The calibration spectra obtained from the Na²² source during flight are used in a computer program to correct for minor drifts and to make the data from an entire flight conform to a single energy calibration. Similarly the GIGI gain stability was acceptable until the ambient temperature fell below -60° to -65° C at which time the gain dropped drastically. This is partially overcome by the use of thermal insulation. Switching between phoswich and no phoswich operation (charged particle rejection and charged particle inclusion) in one GASP flight showed that the count rate, under normal circumstances, due to charged particles did not overload the telemetry circuit, nor did it impair the spectral quality. This finding has led to the development of new spectrometer circuit that does not utilize a phoswich.

PROGRESS REPORT

A new GASP circuit, designated GASP-II, has been designed and built by the Electronics Division. This new version uses a cylindrical NaI(Tl) crystal 3 in. in diameter by 3 in. long attached to an RCA 8054 photomultiplier tube, and does not constitute a phoswich. The larger crystal provides increased sensitivity (which varies directly as the surface area), and also increases the weight which is only partially offset by the removal of the phoswich circuitry. The new package weighs 12 pounds, including water-activated batteries, compared with 9 pounds for the original GASP. Additional efforts have been made to improve temperature stability through the use of compensating circuits. A baroswitch is included for altitude determination, making the device independent of radar tracking. The telemetry link and ground equipment are the same as

those used for the standard GASP. Flight testing of the GASP-II is contemplated from Goodfellow Air Force Base in conjunction with the air-sampling flights made from that location. Further studies of the GIGI under low temperature conditions are contemplated at the same time.

GIGI flights have continued on a quarterly basis from Antofagasta, Chile, while the flights from Barrow, Alaska have been reduced to a quarterly basis as of the middle of 1965. Continued efforts are in progress to calibrate the count rates observed by the GIGI flights in relation to air filters exposed simultaneously or relatively close by in time and space. Furthermore, the discrepancies in the shape of the fission-product distribution curves at extreme altitudes (above 70,000 to 80,000 feet) between the GIGI flights and high altitude filter collections are being examined. In the GIGI data, these discrepancies most probably arise from incorrect assumptions in regard to the shape of the background profile due to cosmic-ray interactions which must be subtracted from the gross profile to yield that attributable to fission products. Continuation of flights during this time of decreasing fission-product activity should provide increasingly accurate measurements of this background radiation and its changes in relation to the solar cycle.

CONCLUSIONS

A GIGI flight at Barrow, Alaska on November 12, 1964 identified a discrete layer of radioactivity within the troposphere (centered about 22,000 feet), which was attributed to debris from the Chinese nuclear test of October 16, 1964. This was confirmed by aircraft sampling in the vicinity on that date. This finding is interesting from two aspects as it demonstrates 1) the high sensitivity of the GIGI (and presumably also the GASP) for the detection of fresh debris present in relatively low concentration, and 2) the long-time (27 days) stability of air masses within the troposphere which is generally considered to be a region well mixed by vertical turbulence.

A new spectrometer system, the GASP-II, is ready for flight testing. The balloon program is expected to continue at its reduced pace through the coming year, maintaining a degree of readiness in the event that limited nuclear testing in the atmosphere may be resumed.

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COMPUTER PROGRAMS FOR GAMMA-RAY SPECTRUM ANALYSIS

Donald M. Nelson, Merlin H. Dipert, and Philip F. Gustafson

PURPOSE AND METHODS

A program for the G. E. 225 computer has been written to analyze complex gamma-ray spectra from exposed air filters, soil samples, and other media. These spectra were obtained by NaI scintillation spectrometry using large crystals in conjunction with a multi-channel, digital, pulse-height analyzer. At present, the program handles up to 10 components (radionuclides), each of which may extend over 400 channels. All input is via punched cards, with the spectra punched automatically by feeding the output of an RIDL 400-channel analyzer directly into an IBM keypunch.

The quantities of the various radionuclides present in the complex gamma-ray spectrum are determined by solving the requisite number of simultaneous equations. This method was chosen rather than a least squares solution because it is less complex, needs less computer memory space, and uses less machine time per analysis. Furthermore, in a limited comparison, the accuracy of the simultaneous equation method compared favorably with that obtained by a least squares solution.

PROGRESS REPORT

Basically, the computer must be given a set of standard spectra (reference spectra)—one for each radionuclide being examined—a background obtained with identical energy calibration, and a set of appropriate channel regions (used to reduce the problem of solving up to 400 equations in n unknowns, to the more manageable problem of solving n equations in n unknowns; n normally ranges from 4 to 8 in our case). Along with each standard spectrum a card containing the chemical symbol, the decay constant, the activity of the standard, and the counting time of that particular standard is read into the memory. The coefficient matrix is calculated automatically before each series of unknown spectra is analyzed so that a change in

analysis conditions between series (such as the addition or deletion of a radionuclide) is easily made. Any number of spectra may comprise a series, but all spectra within a series are analyzed in the same manner.

The first unknown spectrum is then read in together with a card containing the dates of counting and collection of sample, the counting time, and three linear parameters that may be used for weights, volumes, geometry factors, percentages counted, etc. The results are expressed both as count rate on the date of counting and as activity on the date of collection, corrected for the radioactive decay of each of the isotopes involved. If desired, a reconstruction of the initial complex spectrum may be made through the use of the computer results and the standard spectra. The reconstructed spectrum is then printed out together with the observed spectrum and the residual spectrum (i.e., the difference between the observed and reconstructed spectra). The residual spectrum is an indication of the goodness of fit between calculated and observed data, and may be used to determine if a radionuclide not included in the analysis was actually present in the spectrum from the sample in question.

CONCLUSIONS

This program is written to correct for both gain and threshold shifts in the analyzer that was used in collecting the data. This is done by shifting the unknown spectrum so that the various photopeaks present fall into the same channels as they do in the case of the standard spectra. This insures that the same energy calibration is being used for both the standard spectra and that from the sample being analyzed. Other pertinent features include: the ability to impose a smoothing function on the observed spectrum, to calculate ratios of radionuclide activities, and to calculate and analyze a difference spectrum which is obtained from two successive counts on the same sample.

RADIOCHEMICAL PROCEDURES

Sarmukh S. Brar, Jerry R. Kline,* and Donald M. Nelson

PURPOSE AND METHODS

In the course of a gamma-ray spectral analysis of environmental fission and natural radioactivity it be-

came necessary to make chemical separations of the exposed air filter samples. This arose from the fact that tracer Cd^{109} and natural Pb^{210} which are present in air are very low energy gamma-ray emitters, and that their determination by usual gamma-ray spectral tech-

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niques is, at best, difficult in the presence of large concentrations of fission products. In addition, the accuracy of the determination of Be^7 in fresh fission products is questionable because of the presence of Ru^{103} . This complexity arises because the half-lives and energies of the two isotopes are comparable. Chemical separation also permits the determination of Po^{210} which is solely an alpha emitter. An additional method for the determination of various isotopes provides a reliable check on the determination of various isotopes by gamma-ray spectral analysis.

The filters used for the collection of particulate matter in surface air are HV 70 (Hollingsworth-Vose) asbestos-base filters. The chemical procedures described briefly below are especially adapted for the determination of Be^7 , Cd^{109} , Po^{210} , and Pb^{210} in these filters. The air pumps on which the filters are exposed are located on top of the Biology Building.

PROGRESS REPORT

The Chemical Procedure

A portion of a filter composite to be analyzed is digested in hot concentrated HCl with the addition of appropriate carriers. The undigested residue is separated by filtration and saved for later checks on completeness of the digestion. The filtrate is partially neutralized and Pb, Po and Cd are precipitated from the

moderately acid solution as sulfides. The mixture is again filtered and Be is precipitated from the filtrate as the hydroxide and is later separated. The hydroxide precipitate is not a pure Be salt but contains other isotopes as well. The separation is clear enough so that the various gamma-ray peaks it contains are easily resolved by gamma-ray spectral analysis. If desired, Be can be separated from the other isotopes.

The sulfides are dissolved again in HCl and filtered. Po is spontaneously deposited on a nickel disk from 3 M HCl solution and alpha counted. Antimony carrier is added to the solution and Sb, Pb, and Cd, respectively, are deposited from this solution onto platinum electrodes by controlled-potential electrodeposition. Antimony is taken out of the solution; otherwise, it contaminates the deposits of Pb and Cd. As Pb and Cd tend to alloy with platinum, they are deposited onto electrodes which have been previously coated with a thin deposit of copper. The platinum electrodes are then stripped of the depositions and gamma-ray counts of the depositions are made.

CONCLUSIONS

These chemical procedures have been tested and are now being routinely used to make possible the measurement of several atmospheric radioisotopes not measurable by other means.

POTENTIOSTAT

Sarmukh S. Brar and William J. Eisler

PURPOSE AND METHODS

The chemical procedure developed and described in a separate article⁽¹⁾ called for the constant-potential electrodeposition of some isotopes, such as Pb^{210} , Cd^{109} , and Sb^{125} which are present in environmental radioactivity and are collected on air filters.

At the beginning, a manually-operated direct current source using a storage battery served the purpose of developing the technique and proved its feasibility for controlled potential electrolysis. Since manual control is crude and tedious and requires the constant attention of an operator, we decided to build an instrument which automatically performs the function of maintaining the potential of a working electrode constant at any set value. A saturated calomel reference electrode is used in the performance of the electrolysis.

A literature search led us to design and build an instrument which is described here. It is based essentially on the design of a Lingane-Jones potentiostat⁽²⁾ except that the instrument is updated by the use of

solid-state components and improved metering circuitry.

PROGRESS REPORT

The servo-system incorporates a direct-current power supply and integrated switching circuitry which provides voltage for instantaneous braking of the motor. The motor used is a Type K2, 0.7 rpm, reversible, which is manufactured by Bodine Electric Company. The speed of the motor has been geared down to one-half of its nominal value in order to reduce the hunting tendency of the servo-system.

The control circuit dry-cell battery which provides the reference voltage has been eliminated and a two-volt Zener diode regulated power supply is substituted in its place.

The control sensitivity of the instrument is determined by a 5-0-5 microampere meter relay. The internal resistance of the meter relay used is 4300 ohms which gives it a sensitivity of approximately ± 20 mV.

FIG. 203.—Circuit diagram of the potentiostat

FIG. 204.—Photograph showing the potentiostat and electrodeposition setup

Figure 203 shows a complete circuit diagram of the potentiostat and Figure 204 shows a photograph of the experimental set up. Output voltage available from the power supply is approximately 25 V at 4 amperes. A list of major components involved in the construction of the unit is given below.

- MI Meter Relay, range 5-0-5 microamps dc, taut band, double set point, Assembly Products Inc., Model 503L.
- M1A Control Module, double set point for optical meter relay, Assembly Products Inc., Model 903A.
- M Motor, fractional horsepower, Type K2, 0.7 RPM, reversible, Bodine Electric Co. (includes start capacitor C4, 0.8-0.9 mfd 220 volts ac).

- T₁ Transformer, Stancor Transformer Co., Type RT 204.
- T₂, T₃ Variable, Autotransformer, panel type, 1.5 amp, Standard Electric Co., Type 100 BU.
- RY1, RY2 Relay, 115 volt, 3 PDT, Potter and Brumfield Co., Type KRP14A (shown wired with coils in the active state). Both relays pull as soon as power is applied in the automatic position.

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LOW BACKGROUND SHIELDS

Sarmukh S. Brar, Philip F. Gustafson, and Donald M. Nelson

PURPOSE AND METHODS

In order to measure very low gamma-ray activity, measurements must continue for several hours or days, depending on the amount of radioactivity in the sample

and in the natural environment (background). ⁷ Shielding of a detector from these background radiations is usually required and is done by the use of iron, lead, mercury, and/or other shielding materials which

are essentially free of natural and man-made radioactive contamination. When experiments are performed near a reactor facility a need to guard against gaseous effluents, such as Ar^{41} , from the reactor becomes a necessity. In order to overcome some of these difficulties, iron shields were designed and built and are now in use.

PROGRESS REPORT

The shield consists of a rectangular iron box with outside dimensions of 48" x 42" x 36". The walls of this box have a thickness of 6". In addition, the shield is lined inside with $\frac{1}{2}$ " Pb and $\frac{3}{32}$ " of Cd and Cu respectively in order to reduce the low energy continuum and to minimize the effect of any x-rays produced in lead. The unique feature of this shield is that the box is welded (with low radioactivity welding rods) from outside and the door has a built-in gasket which makes a seal onto a tongue on the face of the shield when the door is closed. This procedure makes the shield reasonably airtight for most work. For very low activity work, the shield can be evacuated and filled with a low-activity gas such as CO_2 , aged air, etc., and pressurized slightly above atmospheric. This method not only excludes the reactor effluents from the detector but also eliminates radon and thoron gases

which are always present in air in varying amounts. Consequently, it tends to give stability to the background and allows one to measure lower activity samples than otherwise would be possible.

The detector used in our case is a low potassium content 4" x 4" NaI(Tl) crystal jacketed with $\frac{3}{32}$ " "clean" stainless steel. Such a jacket not only acts to prevent any internal changes in the crystal assembly under repeated evacuation and pressurization but also acts as a beta shield while measuring gamma rays in the presence of beta radiations. The background obtained with this system is 206 ± 5 cpm from 0.01 to 2 MeV over a 200-minute counting period. The only peak which is clearly evident in the background spectrum is that at 510 keV resulting from annihilation radiation. A faint peak is also discernable at 1.46 MeV due to potassium present in the phototube, preamplifier components, etc. The sodium iodide crystal has a resolution of about 8 percent for Cs^{137} gamma-ray energy of 0.662 MeV.

CONCLUSIONS

At present, two shields are in routine use. They have proved quite satisfactory in excluding air-borne radioactivity from the system whenever they are pressurized with a "clean" gas.

EDUCATIONAL ACTIVITIES IN RADIATION BIOLOGY IN COLLABORATION WITH THE OFFICE OF COLLEGE AND UNIVERSITY COOPERATION

I. TELE-LECTURE SERIES IN RADIATION BIOLOGY

During the past year a series of seventeen lectures has been prepared and taped by members of the Division of Biological and Medical Research. These tapes are sent, upon request, to various colleges and universities who may use them as they wish—in courses, seminars, science clubs, etc. Many of the colleges then arrange to participate in a telephone conference to ask questions of the lecturer. These conferences now are held, with four to eight campuses on each conference call, directly from the Biology Building. During the past six months, twelve such conferences have been conducted with twenty-three liberal arts colleges in the midwest. The tapes are prepared for class use and run approximately forty-five minutes in length. With each taped lecture, a printed booklet is supplied each student. The booklet contains a summary of the lecture, appropriate references, and reproductions of the lecturer's slides. It is the object of this program to produce a series of modern lectures in radiation biology which can be used to supplement the standard biology courses presented to juniors and seniors in colleges and universities. A list of the tapes currently in use includes:

SERIES A			Number of Tapes
Lecturer	Subject		
Robert L. Straube	General radiobiology and basic principles	3	
Theodore N. Tahmisi- sian	Effect of x-irradiation on tissue and organelle dif- ferentiation	3	
Herbert Kubitschek and Richard E. Ecker	Biochemical basis of hered- ity, mutations, and cellu- lar control mechanisms	3	
SERIES B			
Howard H. Vogel, Jr.	Some biological effects of external radiations	3	
Philip Gustafson	Environmental radiations	2	
Charles Ehret	Some fundamentals of radi- ation biology	1	
Solon Gordon	Light and chromosome dam- age	2	
SERIES C (In preparation, September, 1965)			
Warren Sinclair	Synchronized culture of mammalian cells and radi- ation studies		
Samuel Leshner	Normal cell cycle in the mouse intestine; the ef- fects of irradiation on the cell cycle; uses of tri- tiated thymidine		
Agnes Stroud	Tissue cultures and chromo- some karyotypes; the use of computers in cytology		

II. SUMMER INSTITUTE

A summer Institute in Radiation Biology was offered to twenty-two college teachers of biology from June 14, 1965 to August 6, 1965. The candidates were selected by Howard H. Vogel, Jr., Technical Director of the course. Most of the participants had a master's degree and about half had a Ph.D. degree. The course was given cooperatively by members of several Argonne Divisions. Fifty-five staff members of the Biological and Medical Research Division participated. Three lectures and a laboratory each day were offered for the

eight-week course. Nine staff members from other divisions and four guest lecturers also participated. Each student also carried out an individual laboratory project under the guidance of an Argonne Laboratory staff member. A list of the candidates and their academic affiliations may be found in Table 100 (See also Figure 205.). This Institute in Radiation Biology was sponsored jointly by the National Science Foundation (participant's expenses) and the USAEC (operating costs).

TABLE 100. CANDIDATES IN ATTENDANCE AT THE
RADIOBIOLOGY INSTITUTE, JUNE 14, 1965-AUGUST
6, 1965

Candidate	Academic Affiliation	State
David Brittain	Ripon College	Wisconsin
John Buegel	Concordia College	Minnesota
Raymond Brand	Wheaton College	Illinois
Wayne Clements	Newark College	New Jersey
J. T. Coleman	Southern University	Louisiana
Jack Denning	Mundelein College	Illinois
Robert M. Dixon	Bay Path Jr. College	Massachusetts
Arthur Johnson	Hendrix College	Arkansas
Vercia Lacey	Tyler District College	Texas
David Ledney	University of Notre Dame	Indiana
Donald Levine	Paterson State Col- lege	New Jersey
Philip Levine	Drake University	Iowa
Montague M. Oliver	St. Joseph's College	Indiana
Jobe Payne	USAEC	Illinois (audi- tor)
Gladys Robinson	George Williams Col- lege	Illinois
George H. Schneider	Quincy College	Illinois
Albert J. Smith	Judson College	Illinois
Sidney R. Smith	Morehouse College	Georgia
Sister M. E. Silkey	Mt. Mary College	Minnesota
Larry Tiezen	Albion College	Michigan
Sister Vincent Kirk	St. Francis College	Illinois
Jack S. Wood	Western Michigan University	Michigan

FIG. 205.—Twenty-two college teachers, members of the Institute in Radiation Biology, conducting an experiment on the irradiation of mammals with fission neutrons at the CP-5 reactor. Dr. Vogel is explaining the gamma-neutron radiation chamber, while Birk and R. Barhorst are helping students load mice into irradiation cages.

III. WORKSHOPS IN RADIATION BIOLOGY FOR FACULTY MEMBERS

During the past year, six college faculty workshops were held at Argonne (on Fridays and Saturdays). Thirty-seven college and university faculty members attended. Among the workshops given were:

Experiments in protection and recovery
Organ weight loss after irradiation

Biological experiments at a nuclear reactor
 I^{131} uptake in rats
Bone-marrow transplantation
Radioautography
 P^{32} uptake in plants
Tritiated thymidine and ascitic tumor cells

IV. STUDENT EXPERIMENTS IN RADIATION BIOLOGY

Ten instructional experiments were conducted at Argonne with six to eight junior and senior biology majors working on each experiment. Among the participating colleges were: Rosary, Wheaton, St. Procopius, Alverno (Wisconsin), and Hanover (Indiana).

The faculty workshops and student experiment programs were in addition to both the ACM and AMU programs at Argonne.

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