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SEMIANNUAL REPORT BIOLOGY and MEDICINE

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Spring 1961

John H. Lawrence, M.D., D. Sc., Editor
Antreen Pfau, Associate Editor

Donner Laboratory and Donner Pavilion

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BIOLOGY AND MEDICINE SEMIANNUAL REPORT

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Donner Laboratory and Donner Pavilion
University of California, Berkeley, California

John H. Lawrence in charge

March 1961

INTRODUCTION

The Donner Laboratory is dedicated to teaching and to the solution of biologic and medical problems using the knowledge and techniques of the basic sciences, especially physics, chemistry, and mathematics. The Laboratory traces its beginning to the first medical studies with radioactive tracer elements made in 1935 by John H. Lawrence and Joseph G. Hamilton. These investigations were made possible by the pioneering school of nuclear physics founded by Ernest O. Lawrence, and the building of the first usefully productive cyclotron under his direction in 1934.

In 1940, in recognition of the importance of the basic sciences to biology and medicine, the late William H. Donner made it possible to build the first wing of the Donner Laboratory. The initial staff applied its efforts to defense problems, particularly in the field of aviation medicine, while much of the building was used temporarily by the Manhattan Project for wartime nuclear weapons development.

In the postwar period, physical and chemical tools have been applied to many fields of biologic research ranging from the molecular level to that of complete organisms, and including many problems related to human disease. As the Laboratory staff grew, the facilities were expanded correspondingly. Donner Pavilion, a wing of Cowell Memorial Hospital designed for medical research, was added in 1952. In 1954 Mr. Robert Donner, the Donner Foundation, and the State of California contributed a new wing to Donner Laboratory, thus approximately doubling the size of the Laboratory.

Donner Laboratory is the center for research and teaching in biology and medicine in the Lawrence Radiation Laboratory, which conducts its extensive program in these and other fields under contract to the Atomic Energy Commission. Thus, much of Donner Laboratory's activity is conducted on behalf of the AEC and forms a part of its nationwide biomedical program. The Laboratory works closely with the other science departments of the Lawrence Radiation Laboratory and the University of California. There is close collaboration with the staff of the University Medical School in San Francisco and with many outstanding medical groups in the East Bay, including the staffs of the Highland Alameda County Hospital, Cowell Memorial Hospital on the Berkeley Campus, the Children's Hospital of the East Bay, and the Alta Bates Hospital. The Donner-Highland Radioactive Isotope Unit at the Highland-Alameda County Hospital is staffed and operated by Donner Laboratory and Donner Pavilion personnel in collaboration with the staff of Highland Hospital.

An essential part of the Laboratory is the active training program in biophysics and medical physics that is conducted within the framework of the University-wide study program. The Division of Medical Physics, an academic unit of the University of California, was established in 1944. The faculty of the Division of Medical Physics of the University of California consists of a number of staff members of the Donner Laboratory. As part of their academic responsibilities these members offer courses related to biologic and medical aspects of radioactivity, radiation and health physics, radiobiology, and biophysics. These courses are taken by undergraduate students enrolled in the physical or biologic sciences, and by graduate students enrolled mainly in biophysics, medical physics, or bioradiology. As members of the interdepartmental group in biophysics, the faculty supervises graduate student research programs. Some staff members also advise students enrolled in the undergraduate biological physics major program.

Over the last eleven years, 55 graduate students working in the Laboratory have received Ph. D. degrees in biophysics, and seven in medical physics. Nine others are scheduled to receive the doctoral degree in 1960-61. In addition, many postdoctoral research fellows and visiting scientists have participated in the training program at the Laboratory. They come to gain experience in one or more of the specialties of the Laboratory--for example, clinical and biologic use of radioisotopes, radiobiology, biomedical instrumentation, and biophysics or medical physics. This training is an effective means of introducing specialized knowledge into many countries throughout the world.

The Laboratory has, throughout its existence, been continually receiving generous support from many private foundations and individuals. These continue to be a source of funds for our fundamental research effort. The University of California also supports the program in teaching and research. The Regents of the University of California and the State of California Cancer Fund have both provided essential contributions to the building, maintenance, and operation of the Laboratory, but its main source of support is from the Atomic Energy Commission through the Lawrence Radiation Laboratory.

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MYRON POLLYCOVE, M. D. --Consultant in Hematology and Associate Research Hematologist in Donner Laboratory; Research Medicine and Medical Physics.

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EXPERIMENTAL SETUP AND DOSIMETRY FOR INVESTIGATING BIOLOGIC EFFECTS OF HEAVY PARTICLES*

Tor Brustad,† Piero Ariotti, and John Lyman

In 1957, shortly after the heavy-ion linear accelerator (Hilac) was in regular operation, a study was started of the effects of heavy ions on various biologic systems. The initial study dealt exclusively with effects on dry samples exposed in high vacuum.[1-7] As these investigations developed, various improvements were made in the setup. These included provisions for exposing biologic samples in the "wet" state and under controlled gaseous atmospheres.[8-11] The equipment available and the dosimetry of the heavy ions that were used in the later phases of the work with the Hilac are described in this paper.

Beam Characteristics

The heavy-ion linear accelerator has successfully accelerated beams of helium, boron, carbon, nitrogen, oxygen, neon, and argon to energies of 10.4±.2 Mev per nucleon.[12] The beam is pulsed, with an adjustable pulse-repetition rate. In this particular investigation, 10 and 15 pulses per second, with a pulse duration of 2 milliseconds, were used. In certain instances, biologic materials have been exposed to a single pulse or to a few pulses in succession. The pulse duration may be varied. Dose rates as low as a few rads per second and as high as 10^6 rads/sec are obtainable.

The Experimental Setup

The experimental setup is diagrammed in Fig. 1. From the exit port of the accelerator, the beam first enters an "analyzer magnet" where it is bent up to 30 deg relative to the original beam direction to insure homogeneity of particle momenta. The beam then passes through the monitor section, which contains three 1/4-mil aluminum foils that also serve to scatter the beam sufficiently to furnish a homogeneous radiation field at the position of the biologic samples at the very end of the apparatus. Highest beam intensity is obtained when partially stripped ions are accelerated. It is thus usual to accelerate B^{+4}, C^{+4}, O^{+5}, and Ne^{+7}. These ions are completely stripped of electrons as they pass through the foils in the monitor section and enter the target section. The target section consists of an electrically insulated Faraday cup, made of 8-in. i.d. brass tubing with a removable back cover, which facilitates attachment of additional equipment. This cup, the main Faraday chamber, is provided with an extension into the beam pipe, as shown in the illustrations. The beam-defining aperture is located just in front of the main Faraday chamber, and care is taken to prevent the beam from striking the extension of the latter.

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*This paper has been submitted for publication in Radiation Research.

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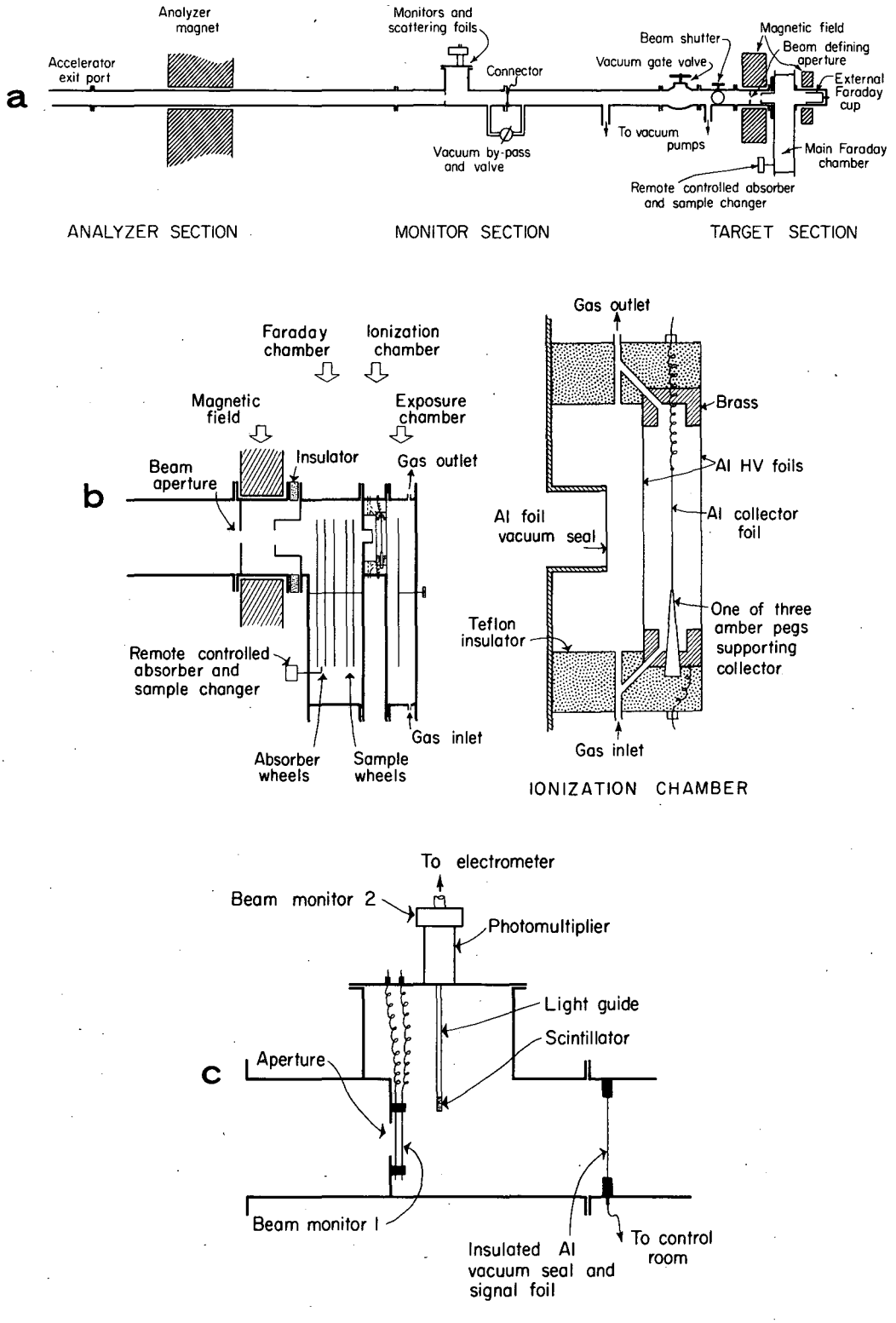

Fig. 1. a. A schematic diagram of the experimental setup, showing the relative positions of (left to right) analyzer section, monitor section, and target section. b. Target section. c. Monitor section.

The beam-defining aperture and the extension of the main Faraday chamber are both located within the field of a permanent magnet arranged perpendicular to the direction of the beam. This field serves two purposes: (a) it prevents electrons that may be in the beam (ejected from the foils in the monitor section) from entering the main Faraday chamber, and (b) it prevents electrons ejected inside the main Faraday chamber from scattering out.

The main Faraday chamber contains three absorber wheels, each with nine aluminum foils of calibrated thicknesses and one blank hole to allow the beam to go through with full energy. This arrangement makes available a large number of independent combinations of absorber thicknesses up to 250 mg/cm^2. The main Faraday chamber also contains two sample wheels, each having eleven sample and five control positions, plus one blank for the beam to pass through with full energy. All five wheels can be rotated independently by remote control, and a panel in the control area shows what absorbers and which sample are in the beam path. The wheels are turned by motors having grounded casings, and the rotating shafts pass through the front plate of the main Faraday chamber on O-ring seals. The shafts are made of brass, and a flexible joint is provided by porcelain couplings. When precautions are taken to keep the insulator surfaces clean, the leakage current from the chamber is negligible.

Attached to the main Faraday chamber is another external Faraday cup, which is insulated from the main chamber (Fig. 1a). This cup is guarded by a magnetic field provided by two horseshoe magnets, which can be moved back and forth to test the behavior of the cup. External Faraday cups of different depths were made and tested for escape of secondary electrons. With a magnetic field of a few hundred gauss, a cup depth of 4 in. or more was found very satisfactory, whereas depths less than 2 in. did not give satisfactory results. This arrangement with two cups provides a means for testing leakage between the cups, from the main Faraday chamber to the motors for the remote controls, through the air surrounding the chambers, with rf on, and with rf on and beam hitting the gate valve in front of the chambers. With proper alignment of the beam, it was found that the readings from the two Faraday cups agreed very well.

The external Faraday cup can also be replaced by a 1- or 2-mil Al window, which makes it possible to take the beam out of the apparatus, facilitating alignment of the setup as well as permitting study of the homogeneity of the cross section of the beam.

For exposure of biologic samples in the wet state and under controlled gaseous atmospheres, an ionization chamber and bombardment compartment were designed (Fig. 1b).

When experiments were performed with the 60-inch cyclotron, the target section was connected directly to the exit port of that accelerator. Homogeneity of the intensity of the beam over the sample area was achieved by combining a 1-mil Al scattering foil upstream with a defocused setting of a pair of focusing magnets. The energy of the protons and alpha particles accelerated in the 60-inch cyclotron is about 12 Mev per nucleon.

Dosimetry

After a beam of homogeneous intensity had been obtained, the beam dosimetry was established by measuring the number N of bombarding particles per cm^2. Knowing this number and the linear energy transfer (LET) in the target material, expressed in units of $Mev/g/cm^2$, the dose D (in rads) is given by

$$D = 1.602\ N\ (LET)\ 10^{-8}. \tag{1}$$

The cross-sectional area and the intensity distribution over the target area were measured in various ways:

(a) At low beam intensity by densitometer readings of exposed Du Pont safety film, type 1290.

(b) At high beam intensity from the radiation-induced colorization of Ozalid paper. Beam areas judged from diameters of burn patterns obtained with the two techniques agreed generally to within a few per cent.

(c) In some cases nuclear track emulsions, mounted perpendicular to the beam in the high vacuum of the main Faraday chamber, were exposed to the heavy ions, and the track density was counted. This technique was also useful for the testing of the edge structure of the beam.

(d) At varying beam intensities, the beam current that passes through a small aperture in the main Faraday chamber, which can be moved across the beam cross section by remote control, can be measured. (This is done by recording it and comparing it with the beam monitors upstream.) The beam intensity coming through the moving aperture can be measured by using either the external Faraday chamber or the ionization chamber. These measurements have shown the intensity distribution across the beam area to be homogeneous within a few per cent when the apparatus is properly aligned. This conclusion has also been borne out in experiments in which dry T-1 bacteriophage has been exposed to different doses. The fraction of the remaining infectivity as a function of dose shows a strict semilogarithmic relationship down to 10^{-6}, which is the lowest survival ratio tested.

When the beam area is known, the dose in terms of number of particles per cm^2 is determined by integration of the beam current as measured by means of the Faraday chambers, with a small correction for charge pickup by the ions, dependent upon the particle velocity.[12] UCRL battery-powered electrometers with negative feedback through accurate load capacitors (or, for current measurements, resistors) were used for these measurements. When samples are exposed on the sample wheels in the main Faraday chamber, the beam is stopped in the discs behind the samples, and the chamber thus provides a direct measurement of the dose the sample has received.

For exposure of samples to heavy ions, with the samples kept at various temperatures during irradiation, or when the effects of various gases were to be studied, the experimental setup shown in Fig. 2 was used. It was found that the main Faraday chamber

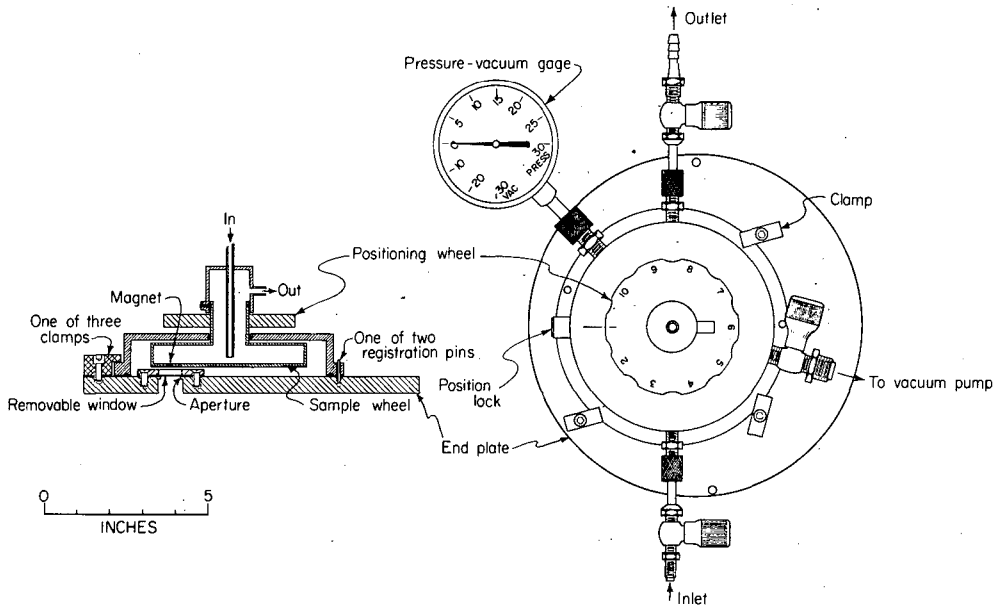

Fig. 2. Bombardment chamber used for exposure of samples (a) in vacuum, (b) in controlled gaseous atmospheres, and (c) under different temperatures.

reading remained unchanged with this chamber attached, regardless of whether the latter was kept at high vacuum as a part of the apparatus high vacuum, or separated by an aluminum window and kept in various gas atmospheres. The doses delivered to the biologic samples were in all cases determined from the main Faraday reading, with a slight empirical correction introduced when the Al window was in place. This was necessary because electrons were knocked out from this foil and hit the samples. This correction was determined for various biologic consequences by exposing samples with and without the Al window in place. As a general rule, the correction was found not to exceed 5%.

Beam Monitors

The monitor section contains three different monitors, as shown in Fig. 1c. Beam monitor I consists of two electrically insulated 1/4-mil aluminum foils set about 2 mm apart, mounted directly on the backside of a grounded beam aperture, to ensure centering the beam in the beam pipe. When a positive voltage is applied to the foil downstream, an enhanced positive signal is obtained from the first foil. This signal depends upon the potential between the two foils and on qualities of the radiation used. Figure 3 shows the monitor current relative to the external Faraday chamber current as a function of the voltage between the two foils. It is seen that the saturation curves obtained resemble the response of an ionization chamber, but the underlying mechanisms are quite different. The monitor was always operated with a potential on the saturation plateau, usually +45 volts. Because this monitor is not stable over long periods of time, and also is to some

extent dose-rate-dependent, its response relative to that of the Faraday chambers was checked frequently.

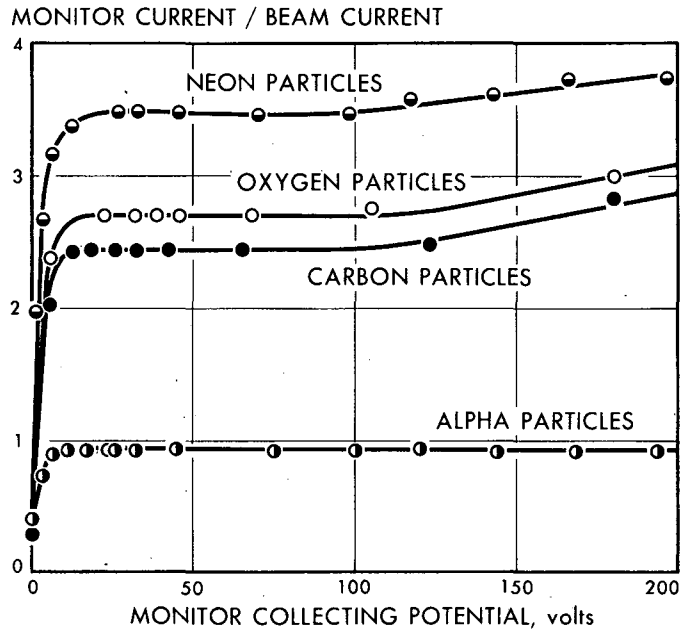

Fig. 3. Representative monitor saturation curves.

Beam monitor II consists of a cylindrically shaped plastic scintillator, 0.5 mm in diameter and 3 mm long, mounted on the end of a glass rod of the same diameter, serving as a light guide. The light guide is connected to a Du Pont photomultiplier tube, No. 6292, and good optical contacts are achieved by using "immersion oil" at the contact surfaces. The light guide is mounted inside a flexible copper tubing. The plastic scintillator is mounted approximately 1 inch from the high-voltage foil of monitor I and in such a position that it is not exposed to the direct beam. Electrons ejected from the Al foil with sufficient energy and at the appropriate angles impinge upon the scintillator. Instead of measuring single pulses, it was found practical to integrate the current from the photomultiplier directly, after the "dark current" was subtracted, by using a balancing countercurrent circuit. The integration was performed as already described for the Faraday chambers.

Beam monitor III is just a single 1/4-mil Al foil that provides a tuning signal for the operators in the control room. The same foil separates the high vacuum in the experimental apparatus from that of the accelerator tank system in order to prevent evaporation products from entering the accelerator system. A vacuum bypass protects this foil until high vacuum is achieved on both sides of the foil.

Ionization Chambers

Measurement of beam current is generally not a satisfactory measure of exposure of biologic samples at low doses. The need thus arose for a more sensitive detector, one

that could be calibrated independently of the Faraday cups, providing a check of the dosimetry already described. Some work was done utilizing a bolometer,[7] but the main effort was devoted to the design and testing of ionization chambers.

The pulsed nature of the Hilac beam imposes certain restrictions on the design of such a chamber. In addition, shallow penetration by the beam presents problems. It was decided that the ionization chamber should have the capacity to measure dose rates as high as 3000 rads/sec with a collection efficiency of 98% or better. With a pulse duration of 2 msec and repetition rate of 10 per sec, this corresponds to dose rates, during the pulse, of 1.5×10^5 rads/sec. From Boag's analysis[13] the collection efficiency of a plane parallel ionization chamber (f) is given as

$$f = \frac{2}{1 + \sqrt{1 - \xi^e}} , \tag{2}$$

where ξ is

$$\xi = \frac{m\, d^2 \sqrt{q}}{V} , \tag{3}$$

m is a constant characteristic of the gas in the chamber, d is the distance between the high voltage and collector electrode, V is the collecting voltage, and q is the ionization intensity.[13] Putting in the necessary constants, one finds

$$V \text{ (in volts)} = 2.05 \times 10^4\, d^2 \text{ (in cm)}. \tag{4}$$

The necessity for keeping the spacing of the plates short is apparent. In other words, with a 1-mm spacing, the required voltage for 98% collection efficiency is about 200 volts. With such a short separation, special precautions would have to be taken in the design of the chamber to facilitate the determination of its effective volume. A chamber design by Boag[14] for fast electron dosimetry was modified to fit the requirements.

Figure 4 shows the design of the ionization chamber and the bombardment chamber attached to it. Ionization chambers were made with various spacings from 5 mm to 0.8 mm between the collector and high-voltage electrode. The chambers were designed with two hv foils and a collector in the center, arranged perpendicularly to the beam. Just in front of the ionization chamber was a 1-mil Al vacuum window to permit exit of the beam from the high vacuum of the apparatus. The distance between the chamber and sample is small, to reduce unnecessary absorption. The spacing is adjustable to maintain the high vacuum as near to the chamber as possible. The beam-defining aperture for the ionization chamber is located as close as possible to the ionization chamber; its maximum diameter is 14.29 mm, but discs with aperture holes of any smaller size can be inserted in the same position. If the area of the aperture is A (in cm^2) and the distance between the high voltage foils is d cm, the effective volume of the chamber is A d cm^3. Brass rings, shaped as shown in Fig. 4, were made to support the three foils. The two hv-foil supporters fit into each other and are screwed together. They are made with great precision to insure a constant foil separation, d, which can be measured with a micrometer screw to an accuracy of 1% or better. The collector foil is mounted on a thin brass ring, supported by three

amber pegs to insure negligible leakage of currents from the hv foils to the collector. This design proved highly satisfactory and eliminated the need for any guard electrodes.

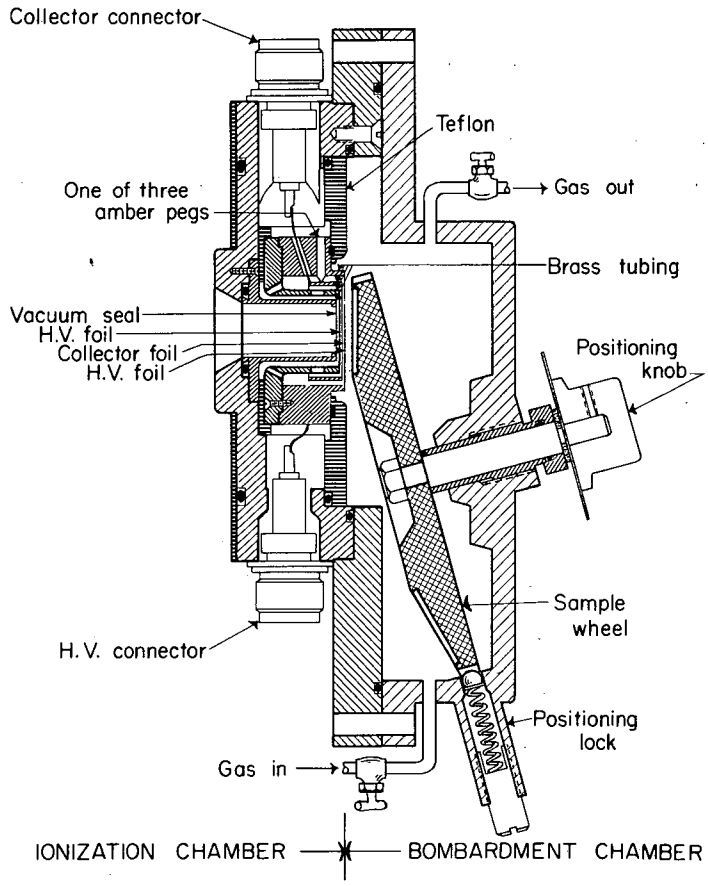

Fig. 4. Design of the ionization chamber and exposure compartment.

A special technique was used to mount the foils on the brass supporters. A foil stretcher was made, similar to one described by Boag,[14] which permits uniform and adjustable stretching of foils of various thicknesses. The foils were stretched tightly until the surface produced a distortion-free optical reflection. The foil was then glued to the brass rings by means of Eastman Adhesive 910. After drying for 10 min, the foil was cut loose from the foil stretcher and trimmed with a razor blade. Maintenance of undistorted reflection properties was required. The foil separation was measured accurately by using a microscope, focusing on the two hv foils separately, and reading off the height difference on the microscope vernier with an accuracy of 0.1%. Usually 1/4-mil Al foils were used, but for testing purposes, 1/10-mil Al foils were also used. An ionization chamber was made with Al-coated Mylar about 0.8 mg/cm^2 thick, with the Al coating a few hundred Å thick. The collector foil was coated on both sides, and the hv foils were coated only on the inner side. This chamber has the advantage of being more nearly equivalent to tissue, and the characteristics of the chamber are being tested.

For routine use the entire chamber is flushed with dry N_2, but tests are also performed with purified argon.

When the intensity distribution of the bombarding particles is homogeneous over the beam cross section, the dose at the position of the ionization chamber is given as[15]

$$D = \frac{QWSm' \times 10^5}{V\rho} \quad , \tag{5}$$

where ρ is the density of the gas in the chamber at the existing temperature and pressure, D is the dose in rads, Q is the charge in coulombs collected from the chamber, V is the collection volume in cm^3, W is the energy in ev per ion pair for the gas in the chamber, and Sm' is the mass-stopping power of the target material relative to that of the chamber gas. In order to determine the dose delivered to samples at a certain distance from the chamber, a correction factor must be applied[15] which, under the present experimental conditions, is not significantly different from the Bragg ratio, when Sm' is taken as the value for particles having the same energy as occurs in the actual sample. Values of Sm' and W as a function of the heavy-ion energy are taken from Birge et al.[15] as equal to that of protons of the same velocity.

Figure 5 shows some typical voltage saturation curves for an ionization chamber having a hv collector-foil spacing of 0.82 mm. Curves are presented for different dose rates. Normally this chamber was operated at 160 volts. Current from the chambers was measured, or integrated and recorded as described earlier for the Faraday cups.

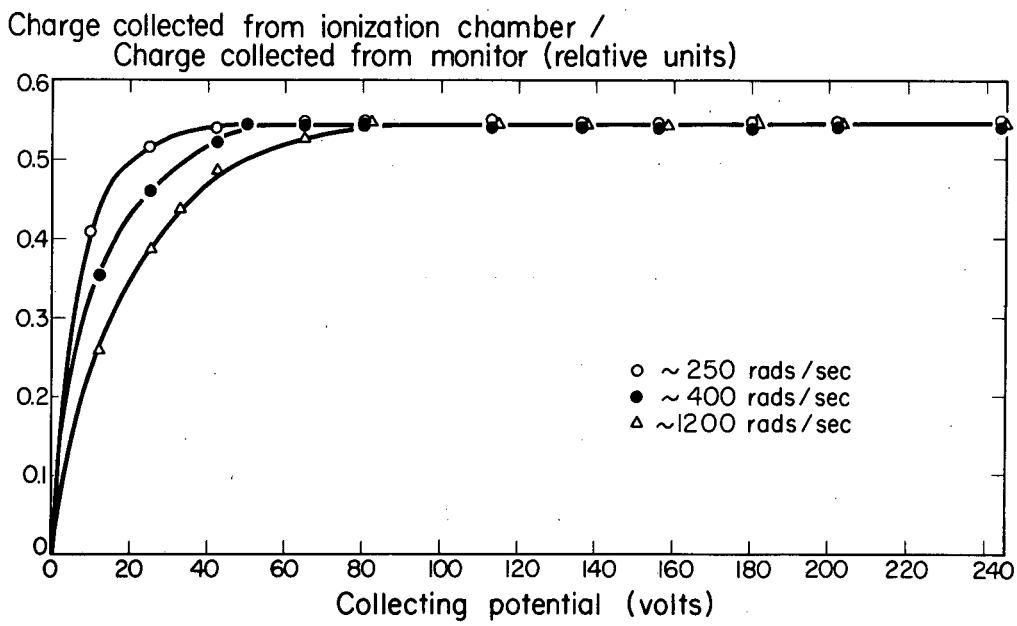

Fig. 5. Representative ionization chamber saturation curves for different average dose rates. The dose rate during a pulse is 50 times as great.

Particle Range and Stopping Power

Introducing Al absorbers in the main Faraday chamber permits measurements of external Faraday-cup readings relative to the monitor readings, and thus provides determination of number-distance curves for the various ions. Connecting an ionization chamber instead of the external Faraday chamber provides "beam Bragg curves," discussed later. Figure 6 shows the external Faraday current relative to the monitor current as a function

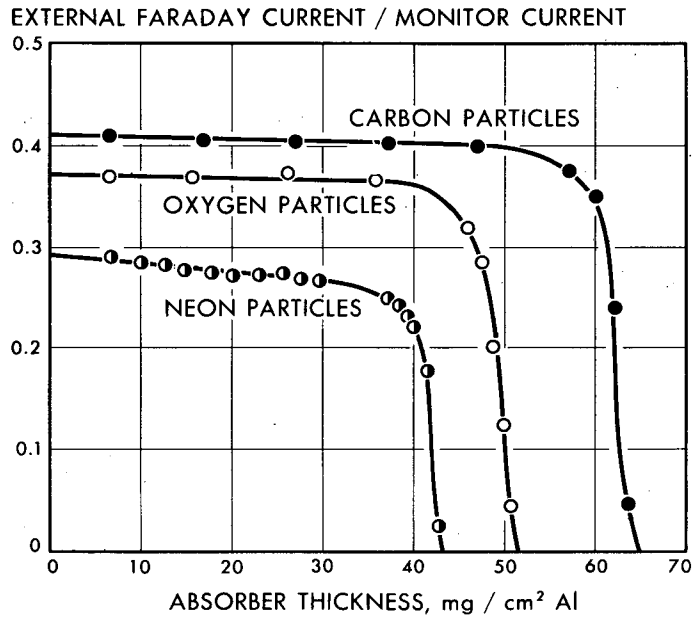

Fig. 6. External Faraday current relative to the monitor current as a function of the thickness of Al absorbers for carbon, oxygen, and neon ions.

of absorber thickness in mg/cm^2 Al. The Ne curve shows a pronounced slope, even for absorbers less than 30 mg/cm^2, which is due partly to charge pickup as the particle velocity is reduced. Correcting for this effect, by using data from Heckman et al.,[12] permits conversion into general number-distance curves. With the range defined as the absorber thickness for which the number of particles is reduced to 50%, (i.e., "mean range") the ranges in Table I have been determined for full-energy particles accelerated in the Hilac and the 60-inch cyclotron.

The Bethe-Block formula does not apply directly to the heavier ions. This is no surprise, because first, the particle velocity is not much greater than that of the fastest electrons in the atoms of the absorber material; second, as the energy of the ions is reduced by penetrating the absorber, the particle velocity approaches that of its own K electrons, and charge pickup becomes pronounced. These effects tend to extend the range beyond that of the completely stripped ion, for which the simple Bethe-Block formula holds.

Table I. Ranges of accelerated particles

Accelerator	Particles	Measured range in Al (mg/cm^2)	Reported energy (Mev/amu)
60-Inch cyclotron	protons	233	12.0
Hilac	He^4 ions	180	10.4 ± 0.2
Hilac	B^{11} ions	82	10.4 ± 0.2
Hilac	C^{12} ions	62.6	10.4 ± 0.2
Hilac	O^{16} ions	49.4	10.4 ± 0.2
Hilac	Ne^{20} ions	42.4	10.4 ± 0.2

In this investigation it was necessary to know the energy of the particles in the samples and the corresponding LET. Fortunately, range-energy measurements of heavy ions in emulsions were made recently by Heckman et al.[12] To a first approximation, these can be converted into corresponding range in a material P according to the following formula, which applies to ions of identical velocity:

$$R_{P(x)} = R_{E(x)} \left(R_{P(r)}/R_{E(r)}\right), \tag{6}$$

where $R_{P(x)}$ and $R_{P(r)}$ are respectively the ranges in P of the heavy ion (x) and a reference particle (r). $R_{E(x)}$ and $R_{E(r)}$ are respectively the ranges in emulsion of the heavy ion and the reference particle. Range measurements of protons in Al are reported by Bichsel et al.,[16] and data for range of protons in emulsion are given by Heckman et al.[12] Range-energy relations of heavy ions in Al, as calculated from Eq. (6), are shown in Fig. 7. It can be seen that the measured ranges of heavy ions given in Table I all fall within the calculated range for heavy ions of energy 10.4±.2 Mev/amu, as reported by Heckman and his associates to be the full energy of ions accelerated in the Hilac.

Dr. Heckman kindly supplied unpublished numerical data on stopping power of standard emulsion for heavy ions. These were similarly converted to corresponding values of stopping power of tissue by using the following formula, which to a first approximation holds for ions of identical velocity:

$$\left(\frac{1}{\rho}\frac{dE}{dx}\right)_t = S' \left(\frac{dE}{dx}\right)_{Em} \times \frac{1}{\rho_t} . \tag{7}$$

The indexes t and Em refer respectively to tissue and emulsion; ρ is the density, $\frac{dE}{dx}$ is the linear stopping power for a particle of velocity v, and S' is the ratio of the linear stopping power in tissue to that in standard emulsion for a proton of velocity v. If one assumes a tissue composition as that given by Lea,[17] it can be shown that the mean

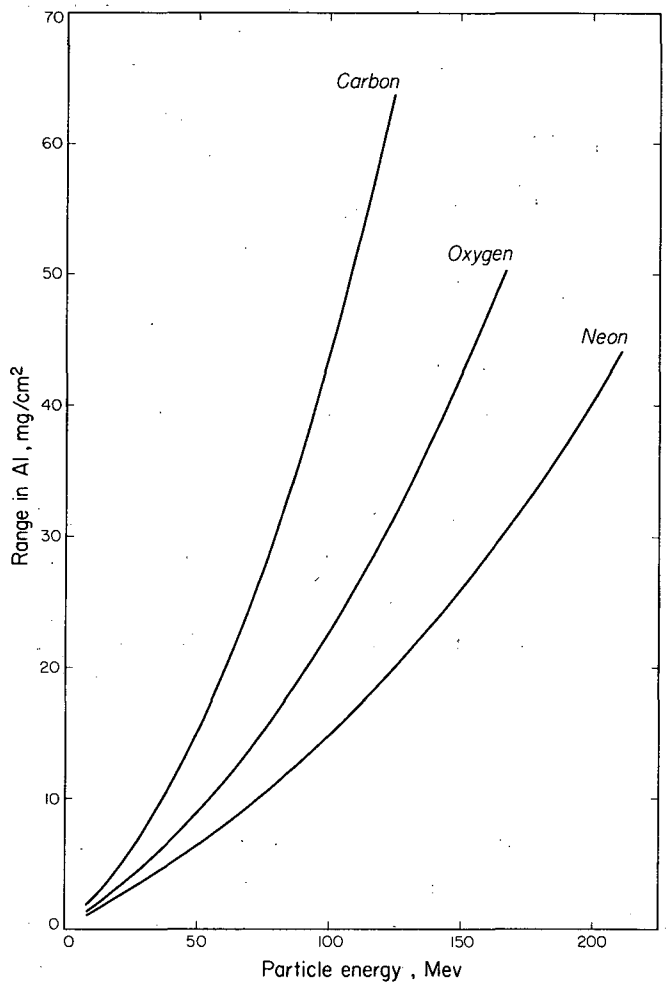

Fig. 7. Range-energy curves in Al of C^{12}, O^{16}, and Ne^{20}.

average excitational potential for tissue is 66.2 ev, compared with 66.0 for water. The effective number of electrons per gram of tissue is 1.1% less than for water. This means that for identical particle velocities, the mass stopping power of tissue is 1.1% less than that of water. This constant conversion factor is taken into account in calculating the stopping power of tissue for protons, from the range-energy data of protons in water reported by Barkas.[18] Equation (7) is then used to calculate the stopping power of tissue for the various heavy ions (Fig. 8).

From this analysis it follows that the range of a particle of velocity v in tissue is 1.1% longer than its range in water, regardless of its velocity. Proton ranges in water are thus converted to corresponding ranges in tissue, and ranges of various heavy ions in tissue are calculated from Eq. (6). Results of these calculations are given in Fig. 9.

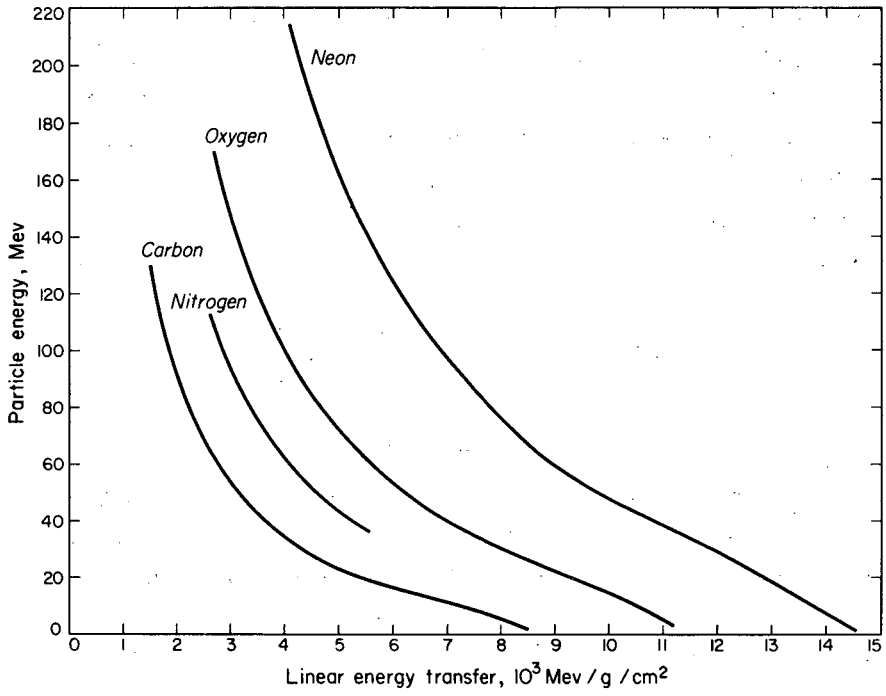

Fig. 8. Stopping power of some heavy ions in tissue as a function of the particle energy.

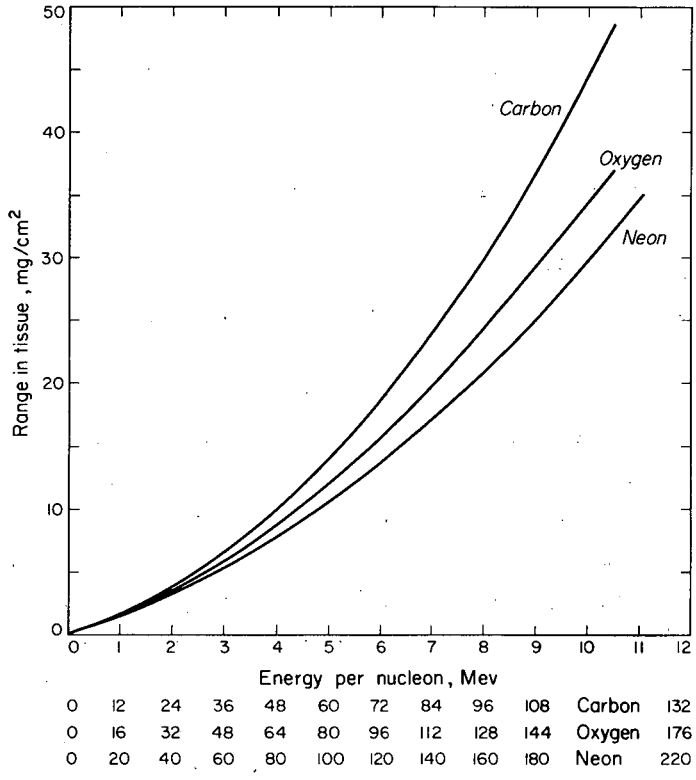

Fig. 9. Range-energy curves of C^{12}, O^{16}, and Ne^{20} in tissue.

To find the stopping power of dry proteins for heavy particles, the chemical composition of casein, given in the Chemical Dictionary as $C_4H_{6.3}N_1O_{1.3}$, has been used as a representative composition for various proteins studied. A similar analysis, mentioned above, shows that the average excitational potential for this compound is 62.7, and that there are 2.68% fewer effective electrons per gram than for tissue. The relative mass stopping power of proteins compared to tissue (Sm') thus is to be calculated for particles of velocity v, using the formula

$$Sm' = \frac{Z_1N_1}{Z_2N_2} \cdot \frac{\ln 2\,mv^2 - \ln I_1}{\ln 2\,mv^2 - \ln I_2}\,, \tag{8}$$

where Z_1N_1/Z_2N_2 is the ratio of the number of effective electrons in proteins to that in tissue, and I_1 and I_2 are the mean excitational potentials of the two substances. Within the particle velocity range studied in this investigation, the correction to be applied in converting mass stopping power of tissue to that of proteins is always less than 2%--dependent, however, upon particle velocity. Curves of mass stopping power of proteins as a function of the energy of the various heavy ions are not shown in this paper.

Bragg Curves for Heavy Ions in Tissue

Bragg curves are obtained by introducing Al absorbers in the main Faraday chamber and simultaneously recording the current from the ionization chamber and that from the monitors. With the monitors calibrated against the Faraday chamber in terms of the number of heavy ions per cm^2, the measurements can be expressed as total specific ionization as a function of distance from the end of the particle range. In a variety of experiments, a knowledge of such Bragg curves in tissue is required. In some experiments--for example, the production of laminar lesion in brain tissue--it was necessary to estimate the dose at the peak of the ionization curve for the particular beam cross section used. This problem is quite complex, and several approximations are involved.

The following procedure was used. All absorbers up to and including half the gas gap and half the collector electrode of the ionization chamber were added up and expressed as mg/cm^2 of Al. The corresponding particle energy in the center of the chamber was determined from the range-energy curves in Al. The range of ions of that energy in tissue was then determined from the range-energy curves in tissue. The relative beam ionization was determined by using an N_2-filled ionization chamber, and the tissue dose was calculated according to Eq. (5). W and Sm', functions of particle energy, were taken from Birge.[15] For low particle energies, there are no accurate values of W and Sm', and theoretical values of Sm' (from formulas such as 7) lead to unreasonable results. Simple extrapolation to zero energy with Sm' = 1.21 and W = 39 ev was used quite arbitrarily. Figure 10 shows the result obtained for four different ion beams. Because of the inaccuracies mentioned above, the peak height also is determined with considerable uncertainty. When plotted (Fig. 10), the area under each curve must equal the total

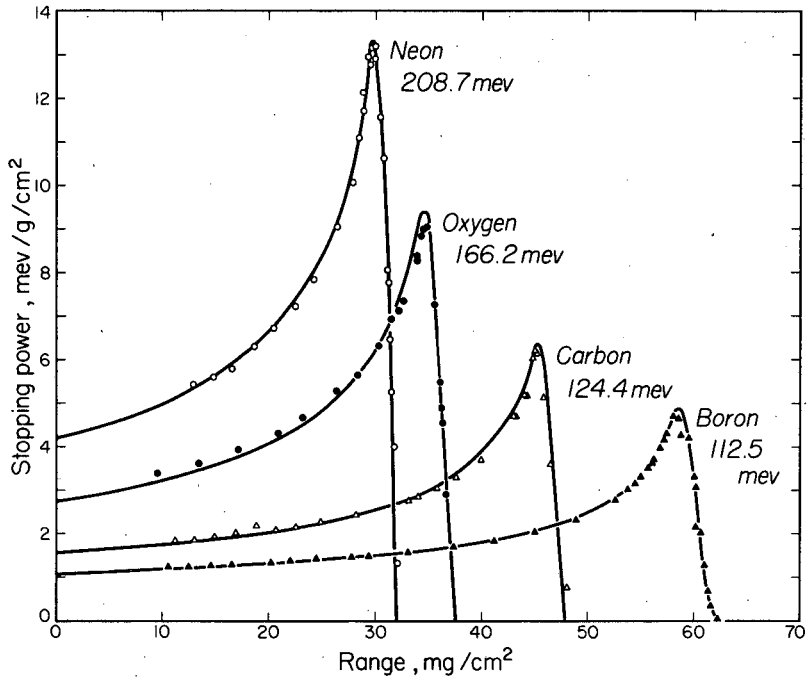

Fig. 10. Bragg curves in tissue of Ne^{20}, O^{16}, C^{12}, and B^{11} of initial energy 10.4±.2 Mev/amu.

particle energy, which is known to be 10.4±.2 Mev/amu. This sets an upper limit to the peak height. Figure 10 also shows the integrated areas, which in all cases are within the experimental uncertainty of the primary particle energy. The solid curve from zero absorber up to a few mg/cm^2 from the peak is converted directly from Fig. 8, while the remaining part of the curve is based exclusively on experimental points.

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FLUORESCEIN AS A SENSITIVE SEMIQUANTITATIVE INDICATOR OF INJURY FOLLOWING ALPHA-PARTICLE IRRADIATION OF THE BRAIN*

Donald C. Van Dyke, Peter Janssen, and Cornelius A. Tobias

The dye, soluble fluorescein U. S. P. (uranine), which is used routinely by physicians as a convenient and sensitive indicator of damage to corneal epithelium, has been found to be an equally convenient semiquantitative and sensitive indicator of irradiation injury to brain tissue. The use of this method to detect damage to brain tissue is based on the same principle as applies in its use as an indicator of damage to the corneal epithelium, i. e., normal brain tissue, like normal corneal tissue, does not stain, whereas injured tissue of both types readily does. The method is quantitative, since the intensity of staining is dependent on the severity of injury and number of injured cells. Permeability of the tissue to fluorescein represents a physiologic or biochemical alteration of the cell that precedes histologic changes demonstrable by light microscopy.

The invasion of brain by tumor tissue, which is permeable to fluorescein, can be recognized by the fluorescence of the tumor following administration of the dye. This phenomenon has been successfully used in a technique to locate brain tumor tissue at surgery.[1]

This paper describes the use of fluorescein staining as a sensitive and semiquantitative indicator of injury to brain tissue following localized irradiation by means of a beam of alpha particles from the 184-inch cyclotron. The method has been used to demonstrate the marked difference in radiosensitivity of brain tissue in different species.

Material and Methods

Male rats of the Long-Evans strain, 28 days of age at the time of irradiation, were used routinely except when the effect of age difference was investigated. Young adult cynamolgous monkeys and rabbits of the New Zealand White strain were used to compare the radiosensitivity of other species. During irradiation all animals were lightly anesthetized with barbiturate, and their heads were confined in specially designed headholders. Figure 1 illustrates the type of holder used for rats. Rigid fixation of the heads of all animals was accomplished by means of ear plugs and a sharp pin inserted into the gingiva and maxilla between the dorsal incisors. It was necessary to use Xylocaine anesthesia locally in the ear canals and gingiva of the monkeys and rabbits in order to keep the level of barbiturate anesthesia low.

Proper positioning of the beam was insured by having an x-radiation source aligned with the path of the beam so that a roentgenogram of the skull with the image of the alpha-particle beam superimposed could be made for each animal. For this purpose a Land

*This paper was presented at the International Symposium on the Response of the Nervous System to Ionizing Radiation, September 7-9, 1960, Chicago, Illinois.

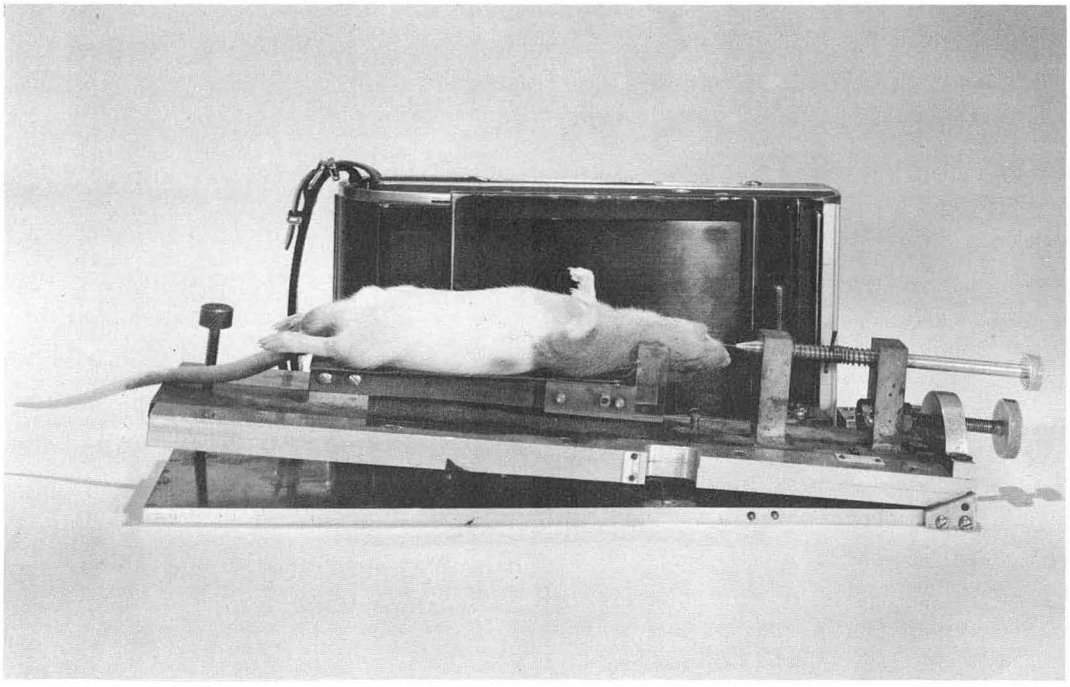

Fig. 1. Anesthetized animal with head mounted firmly in a specially designed headholder. A Land camera is placed in the path of the beam beside the animal's head to position the beam properly.

camera was placed in the path of the beam beside the animal's head (Fig. 1). Figure 2 shows the camera roentgenogram of a rat's skull with the image of the 2.0×25.5-mm beam superimposed. Such pictures are permanent records that may be used later for comparison with the position of the band of fluorescence or the site of histologic changes.

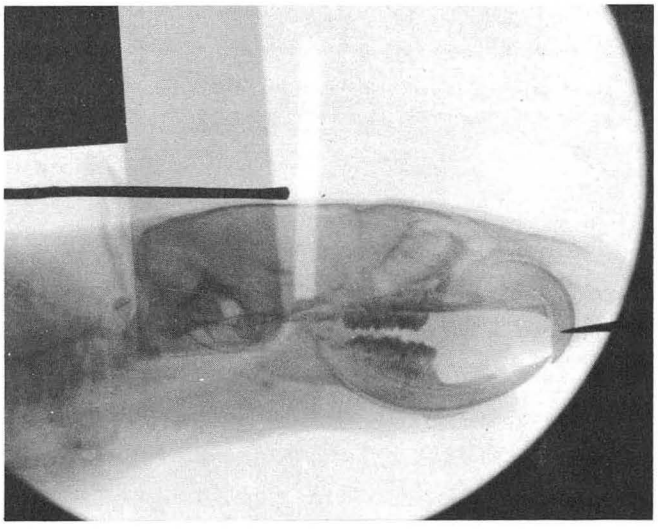

Fig. 2. A Land camera roentgenogram of head of rat with the image of the 2.0×25.5-mm beam superimposed.

A 2.0×25.5-mm brass aperture, 6 in. thick, was used throughout the study except in the experiment in which the effect of the size of the aperture was studied specifically. The distance between the aperture and the area of the brain to be studied was kept constant (1.75 in.).

Fluorescein staining of the damaged brain tissue was accomplished by administering fluorescein sodium (100 mg per ml, pH 8.2) intravenously to animals that were under ether anesthesia (10 mg per 60 g). Forty-five minutes later the animals were anesthetized again with ether. The heads were removed by guillotine and immediately frozen in liquid nitrogen.* Each head was then sectioned on a high-speed bandsaw along the sagittal plane, which was 1.75 in. from the aperture at the time of irradiation. The cut surface was freed of dust fragments by holding it under running water. Care was taken to prevent melting of the tissue. The two frozen halves were then examined in a darkroom under an ultraviolet light. A visual estimation of the intensity of the fluorescent band was recorded. Evaluation of the fluorescein staining was made on the basis of an arbitrary scale from 1+ to 4+, with 1+ being faintly visible and 4+ being intensely so. When subsequent histologic examinations were made, the frozen brain was immersed directly in formalin and processed in the usual way. The skull was opened carefully, and the head immersed in 10% formalin. The brain was removed from the skull the following day and placed in fresh formalin. When thoroughly fixed, the irradiated area was blocked out, dehydrated in graded alcohols, and embedded in paraffin. Sections were cut at 6 microns and stained with hematoxylin and eosin and van Gieson. A 1% thionine solution was used to reveal Nissl substance, and Palmgren's silver-impregnation method for fibers.

The beam used throughout this study consisted of 900-Mev alpha particles from the 184-inch cyclotron.[2] The animals were given a single dose delivered at a dose rate of 2500 rad/min.

The beam was monitored with a parallel-plate ionization chamber, with aluminum foil electrodes and nitrogen atmosphere.[3,4] For absolute standardization this ion chamber was calibrated against a Faraday cage, which was used to measure the particle flux in the beam. The distribution of beam intensity after the beam passed through the aperture was calibrated by means of photographic emulsion dosimetry. The beam, measured by the monitor ion chamber, was allowed to pass through the aperture and fall on a photographic film, exposed in a phantom corresponding in position to that of the head of the irradiated animal. Densitometric evaluation of the photographic film yielded information about dose distribution. Dosages were calculated in rad, and refer to the peak attained in the central plane of the knife-edge lesion.

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*Cytologic studies were made of brain tissue that had not been subjected to freezing, and it was found that rapid freezing with liquid nitrogen did not introduce artifacts that complicated microscopic evaluation of the tissue.

Results

The minimal dose that produced observable changes, and the time of onset of these changes in the brains of rats, were determined by irradiating animals with single doses ranging from 5000 to 26,000 rad and autopsying the rats at postirradiation intervals ranging from 6 hours to 100 days. Fluorescein staining and morphologic changes as seen by means of light microscopy were used as the criteria of irradiation damage. Figure 3 summarizes the results obtained. The time of appearance and the intensity of fluorescein staining are markedly different following different doses. At the lowest doses, 5000 and 6000 rad, no staining was evident until several weeks after irradiation. The intensity of staining was low and the effect appeared to be transient. Following doses of 26,000 rad, the dye was evident 24 hr after irradiation and liquefaction of the irradiated zone was seen by the third postirradiation day.

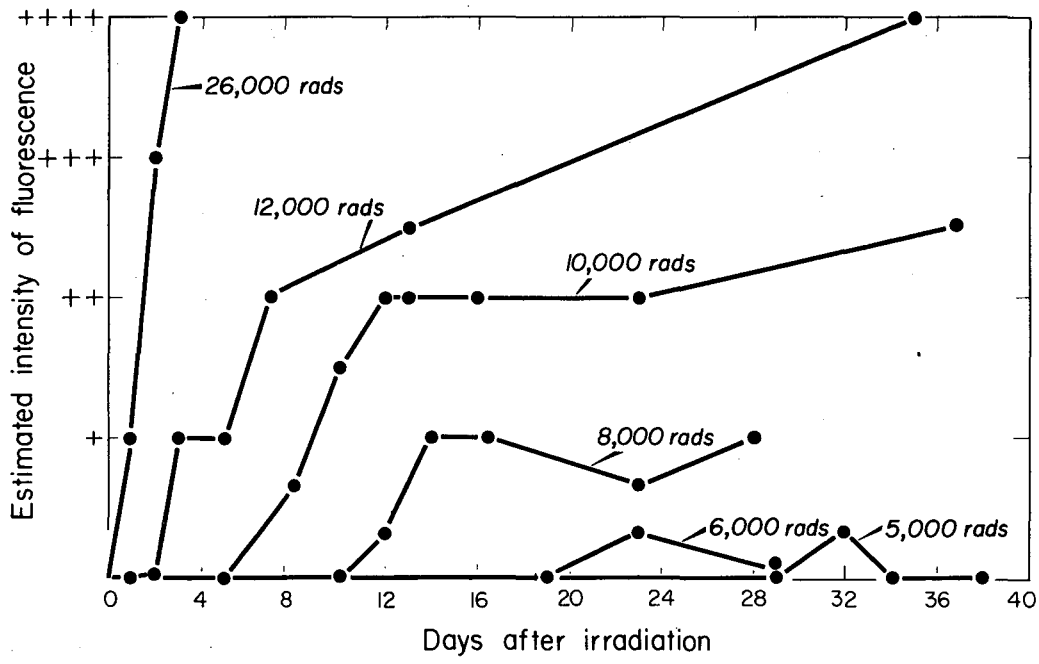


Fig. 3. Summary of results obtained by using fluorescein staining as a criterion of damage in rat brain. The time of onset and intensity of fluorescein staining are markedly different following different doses of radiation.

Radiation doses between these two extremes and differing by 20% can be clearly separated, both as to time of appearance and intensity of staining (Fig. 3). Figure 4 is a plot of the time of first appearance of fluorescence as a function of dose, and illustrates the definite relationship between dose and time of appearance of stain.

Microscopic examination of brains that were examined for fluorescence as well as of those of companion rats from whom the tissue was removed and fixed without previous freezing demonstrated that a lesion is seen earlier and at lower doses by means of

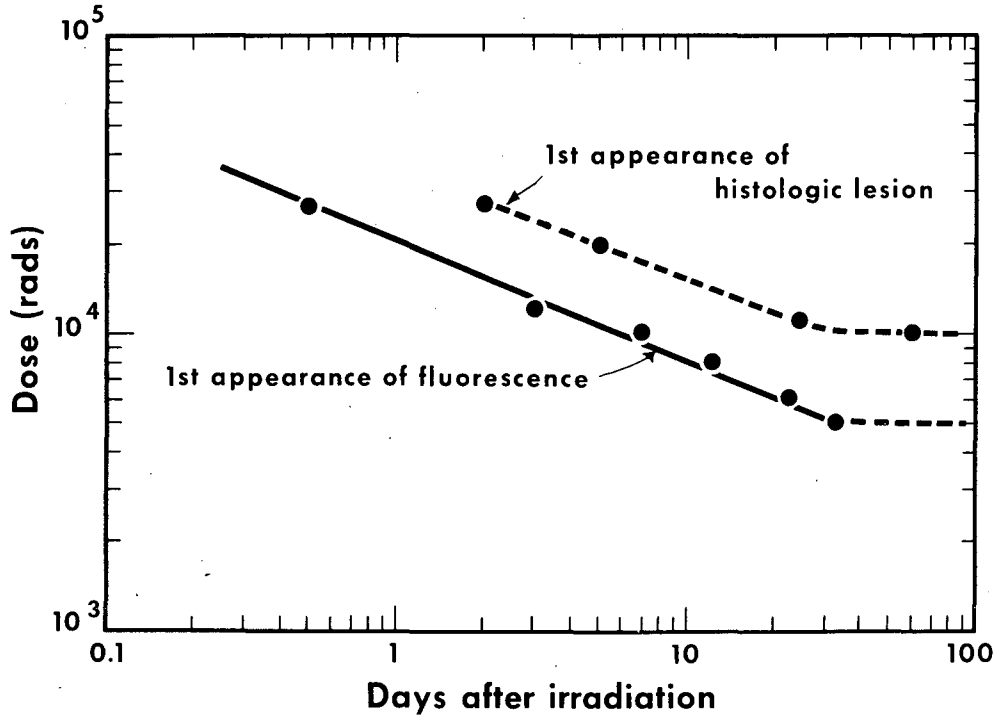


Fig. 4. A log-log plot of the time of fluorescence as a function of dose, illustrating the definite relationship between dose and time of onset of recognizable changes (staining with fluorescein).

fluorescein staining than by light microscopy. After a dose of 8000 rad, a fluorescein-staining lesion appeared during the second and third weeks after irradiation. No subsequent histologic evidence of damage could be seen, no lesion was recognizable microscopically during the first few weeks (none by 37 days), but eventually (84 days or more) a minimal lesion appeared in the irradiated area.

At doses above 10,000 rad, microscopic evidence of a lesion appeared earlier. Following 20,000 rad (6 weeks postirradiation), complete necrosis and removal of the irradiated zone with healing of the margins occurred. At this dose, the first recognizable morphologic changes were seen by the fifth day.

Because doses of irradiation differing by 20% can be differentiated easily by the fluorescein technique, it was thought that this technique might be useful in demonstrating whether such variables as age of animal, size of aperture, or type of particle would alter the result.

A dose of 10,000 rad appeared to be best for demonstrating differences in effectiveness. At this level there is a definite time lag which is followed by a plateau in response. This makes possible a clear differentiation from the response to the next highest or next lowest dose (12,000 or 8000 rad).

The effect of age of the rat on fluorescein staining in the irradiated brain was studied by using 22-day and 120-day-old rats, a dose of 10,000 rad, and a postirradiation interval of 7 days (four rats of each age). The results obtained from the two groups were identical,

three rats of each group being classed as 1+, and one from each group being classed as 2+, a response that could not be expected from a dose effect equivalent to 8000 or 12,000 rad. Thus, the appearance of the fluorescent-staining lesion is not markedly affected by the age of the animal, within the range studied.

The effect of size of lesion on fluorescein staining of the irradiated rat brain was studied by using rats irradiated with 10,000 or 8000 rad through a 4-, 2-, or 0.5-mm slit aperture (2 mm was used in all other studies) and a postirradiation interval of 7 days. The exact dosimetry for the different apertures was determined in the manner described above. When the animals were autopsied, all brains that had been given the larger dose showed a clear band of fluorescence corresponding to the position of the beam. No brain given the smaller dose showed a lesion. The dose-effect relationship thus appears to be independent of the size of the irradiated area within the limits studied, i.e., 0.5 to 4 mm.

Two experiments were done to compare the effectiveness of a given dose of alpha-particle radiation in different species. Rats, rabbits, and monkeys were given a single exposure to 11,000 rad, and representative animals were examined at various postirradiation intervals. In the first series, the earliest time interval was 48 hr, by which time the brain of the monkey showed 4+ fluorescein staining and beginning liquefaction of the irradiated area. Fluorescein staining in the second series, in which the time intervals were shortened in order to obtain early observations on monkeys, is compared in Fig. 5.

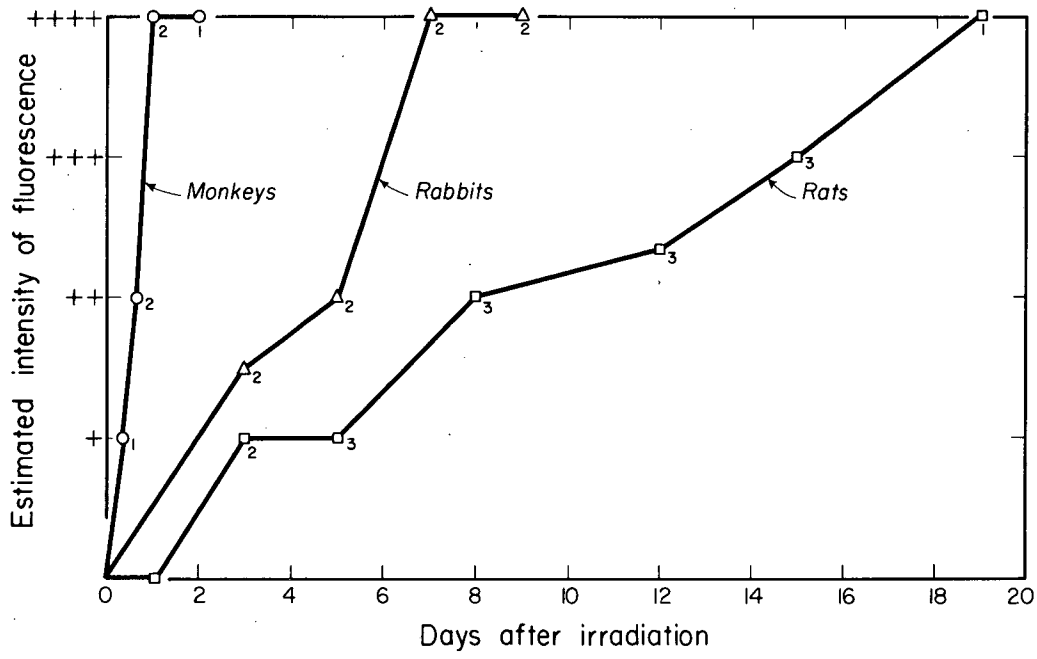


Fig. 5. A comparison of the appearance of fluorescence revealing lesion in the brains of rats, rabbits, and monkeys, given the same dose (11,000 rad) through the same aperture on the same day.

Five monkeys given 3000 rad in the same experiment and autopsied at 3, 5, 8, 12, and 15 days, gave no evidence of staining. By comparing the results in Fig. 5 with the dose-response curve in Fig. 3, one sees that the response obtained from the rats in this experiment corresponds to a dose of approximately 11,000 rad, whereas the response of rabbits corresponds to approximately 15,000 rad, and that of monkeys corresponds to something greater than 26,000 rad. Figure 6a illustrates the intensity (4+) of fluorescein staining of the monkey brain 24 hours after a dose of 11,000 rad. The photograph was taken while the brain was frozen, using ultraviolet light. Rats given the same radiation dose showed no evidence of fluorescein staining at 24 hr and only 1+ fluorescence at 5 days. The monkeys killed at 24 and 48 hr had generalized paralysis and periodic clonic convulsion, revealing that permeability to fluorescein as well as functional capacity of the tissue was altered markedly. Morphologic changes seen in the monkey brain 24 hours after a dose of 11,000 rad (Fig. 7) are compared with those in the rat brain 24 days after the same dose delivered under identical conditions.

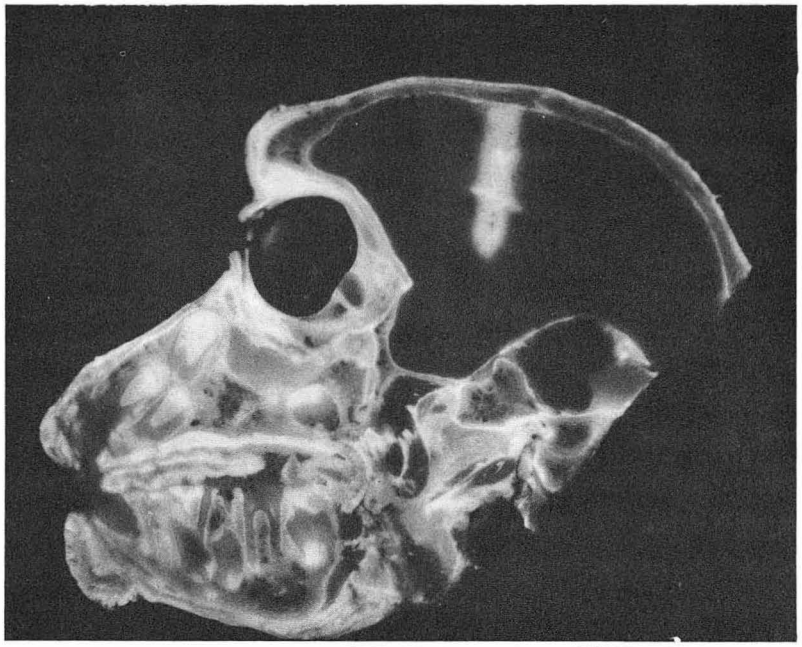
Figure 7a shows a low-power (H&E×9) microphotograph of the irradiated portion of the brain from a monkey that was the companion to that shown in Fig. 6a. The animal was killed at 24 hours after exposure to 11,000 rad. The width and position of the beam is indicated. There was a 6-mm-wide band of intense (4+) fluorescence extending three-quarters of the way to the bottom of the section in the fresh specimen. The photograph reveals the isolated patches of lightly stained tissue seen in the gray matter, and the mottled appearance of the white matter in the path of the beam. Figure 7b shows the lightly staining patches in the gray matter enlarged 32 times. By 48 hr, the entire irradiated area had become liquefied.

Figure 7c shows a section of rat brain 24 days after the same dose and aperture as were used to irradiate the monkeys (Fig. 7a and b). Without magnification or with only low magnification, it can be seen that there is a decrease in staining quality of the ground substance of the cortex, and some capillary dilatation. Figure 7d is a photomicrograph of the area of the cortex showing changes. Microscopic examination of the brains of rats from the same group autopsied at 20 days or less after irradiation showed no recognizable morphologic changes. By 44 days there was marked hemorrhagic necrosis in the irradiated area, with lethal subarachnoid hemorrhage occurring in some animals. The pathologic change was grossly comparable to that found in the monkey 48 hours after irradiation. A detailed study of the histopathology of such lesions has been made by Janssen, Tobias, and Haymaker.[5]

Two methods have been suggested for quantitating more accurately the extent of injury, using fluorescein as an indicator and as a method of evaluating the accuracy of the arbitrary visual grading of the lesions. It is possible to photograph the lesion, using standardized conditions under ultraviolet light (Fig. 6), and to quantitate the film by means of a microdensitometer. Pieces of tissue of uniform size taken from the center of the lesion have been analyzed for fluorescein content by means of a fluorespectrometer.

The values obtained for tissue that had been graded 1+, 2+, and 4+ were respectively 62, 78, and 460 μg of fluorescein.

a



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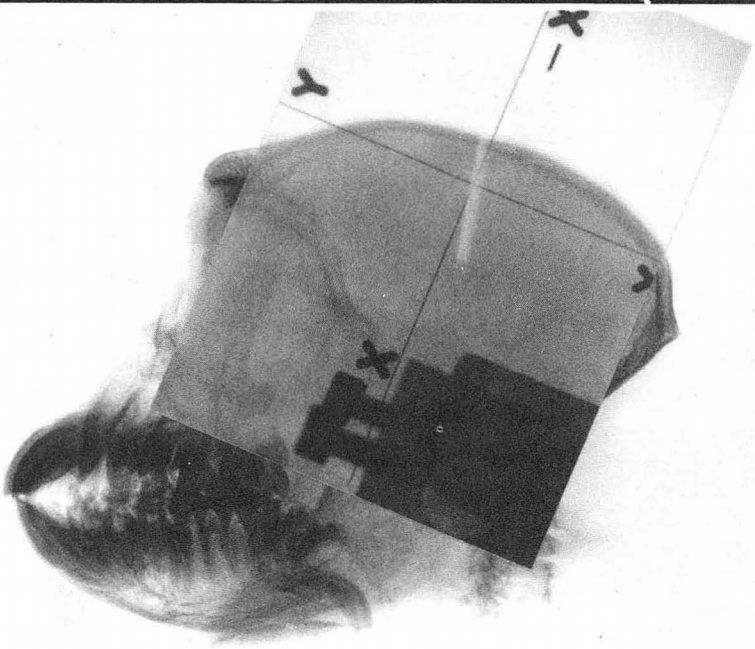


Fig. 6a. Illustration of the intensity (4+) of fluorescein staining of monkey brain 24 hr after exposure to 11,000 rad. The photograph was taken while the head was still frozen. Rats given the same exposure and killed at the same time showed no fluorescence.
b. Land camera picture taken at the time of irradiation superimposed on a roentgenogram of monkey skull for orientation.

Discussion

Fluorescein staining as a criterion of brain injury following irradiation has been described in detail because the method is quick, simple, semiquantitative, and remarkably sensitive for brain, rather than because there is any basic physiologic or biochemical significance of the property of staining per se. The fact that the tissue takes up the dye only after it is injured would seem to indicate that the membranes of injured cells become permeable to substances that they exclude under normal conditions. When considering brain tissue, however, one cannot ignore the so-called "blood brain barrier" phenomenon that has been the subject of so much controversy.[6] This study does not reveal the mechanism involved in acquisition of stainability of brain tissue following irradiation.

It should be emphasized that the results presented in this paper relating to fluorescein staining and morphologic changes were caused by single high doses of irradiation and are, for the most part, confined to observations made during a 3-week period subsequent to irradiation. The possibility that profound changes may occur in the central nervous system many months or years after relatively low single doses of radiation has been explored by others,[7-9] and has not been investigated in this study.

The doses of alpha particles that must be given in order to produce changes in the central nervous system have been found to be relatively high (above 5000 rad). Parallel studies done by Janssen, Tobias, and Haymaker, using alpha particles, confirm the finding that a dose in excess of 5000 rad (7500 was the minimal effective dose found by these authors) must be given before morphologic changes can be seen in the rat at any postirradiation interval.[5] Hicks and Montgomery, however, have shown that at 6 to 24 hr following 1200-rad x irradiation of the head of rats, there are necrotic oligodendroglia cells scattered throughout the white and gray matter.[10]

The greater radiosensitivity of the primate brain than of the rodent brain, as judged by the fluorescein-staining technique and gross or microscopic morphologic changes, has been observed and emphasized by Arnold and his associates[7-9] and by Lindgren.[11] The work of these authors also indicates that the primate brain is more radiosensitive in the early postirradiation period after high doses as well as in the late postirradiation periods after relatively low doses. Lindgren[10] has emphasized that the radiosensitivity of the brain appears to vary from one region to another, cortex and the medullary region immediately underneath it being less radiosensitive than deep-seated parts of the white matter.[12-14] The radiosensitivity of the white matter also appears to vary from region to region, as is suggested by the disseminated appearance of the lesions on uniform irradiation of the entire brain[15] and by lesions seen in the midbrain.[7-9] One must be cautious in generalizing about brain tissue as a whole, and cautious in comparing work done with different types of radiation, different species, and different postirradiation intervals.

Monkeys exposed to 11,000 rad gave a greater response than that found in rats

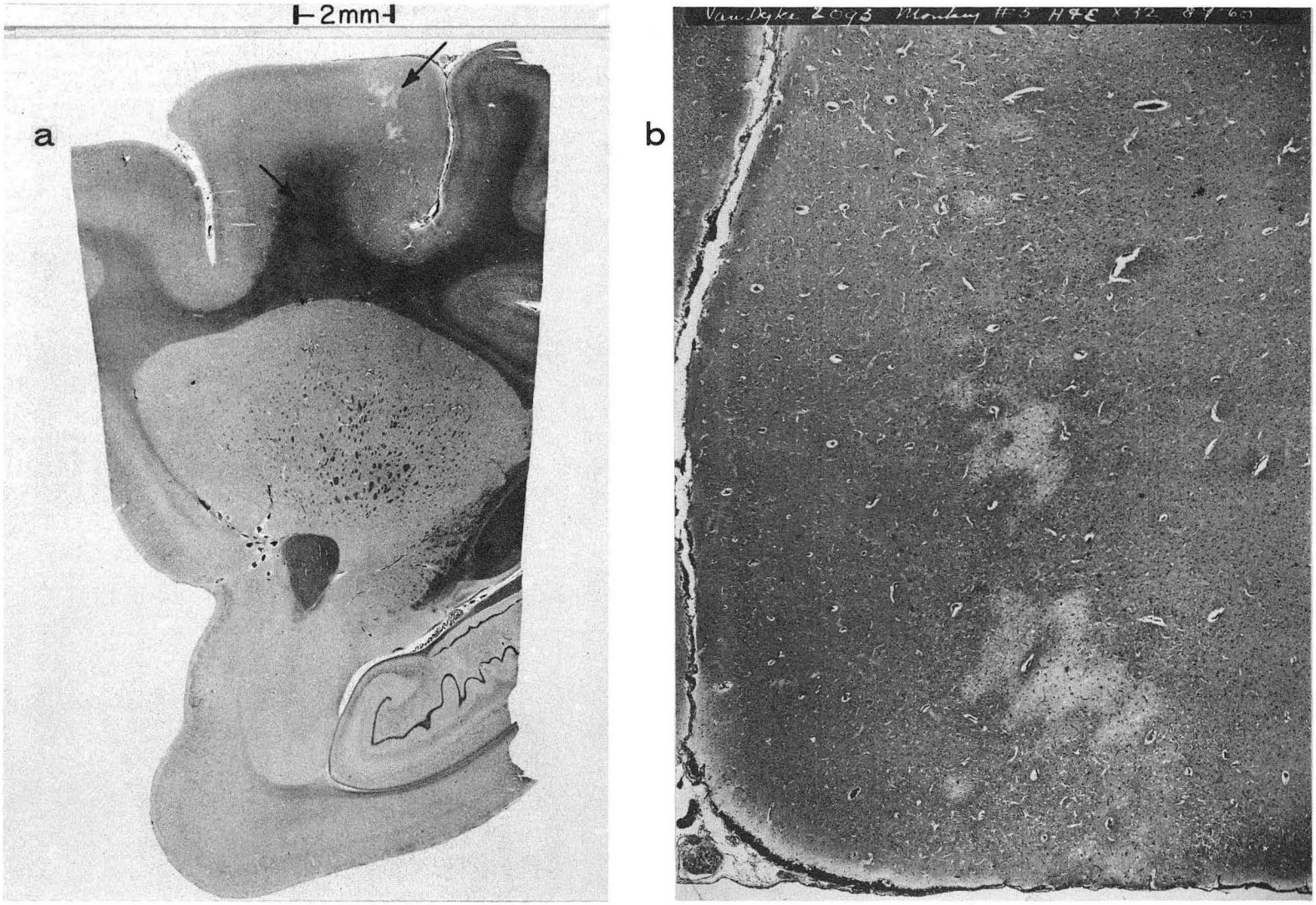
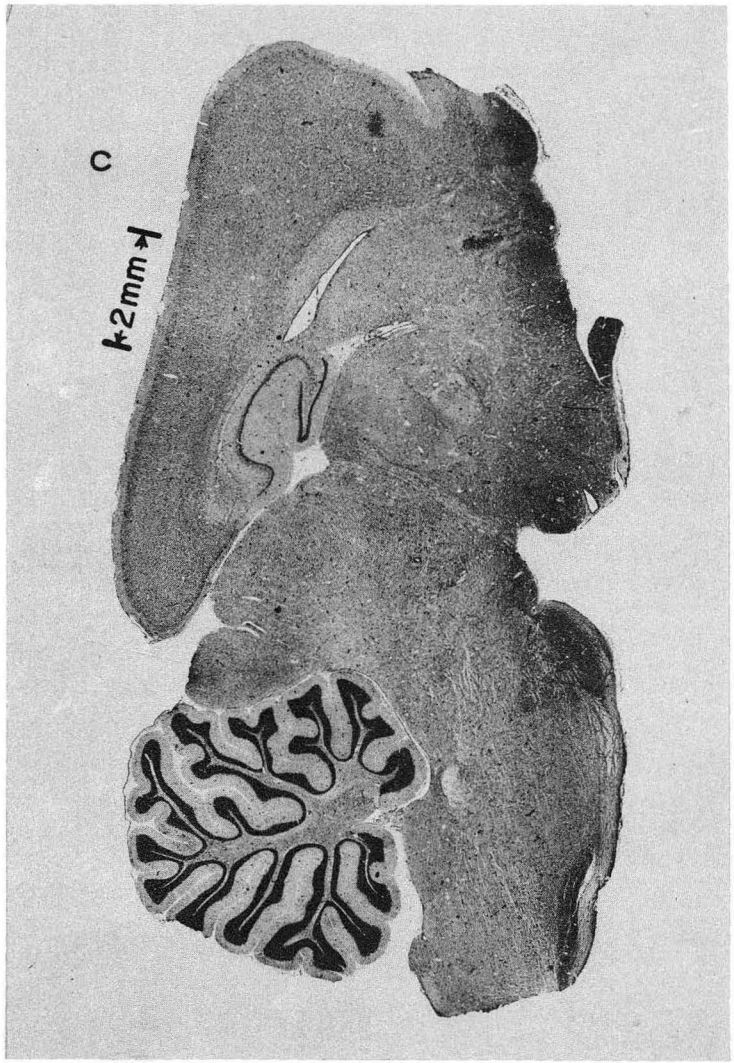


Fig. 7. a. A low-power (H&E×9) microphotograph of the irradiated portion of the brain from companion monkey to that shown in Fig. 6(a). The animal was killed 24 hours after exposure to 11,000 rad. The width and position of the beam are indicated.
b. Lightly staining patches in the gray matter enlarged 32 times. By 48 hr the entire irradiated area had become liquefied.



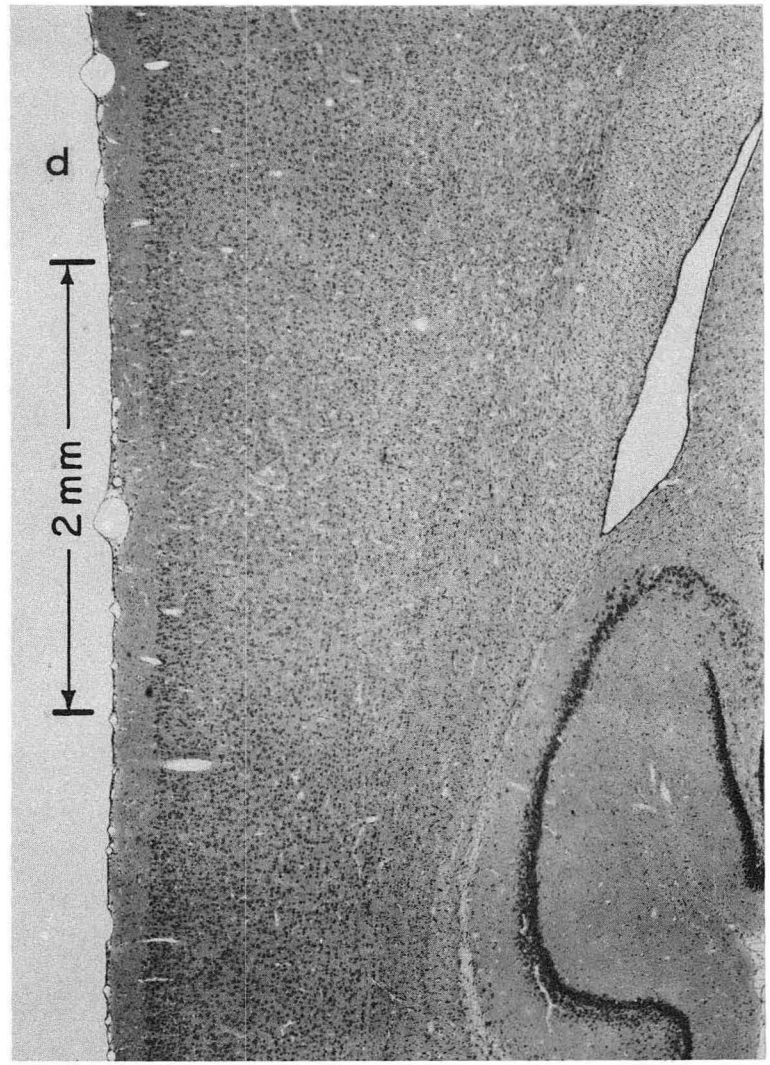


Fig. 7. c. Rat brain (H&E×9.3) 24 days after the same dose and aperture as used for the specimens presented in Fig. 7a and b.

d. Higher-power microphotograph (× 35) of the area of the cortex showing changes. Microscopic examination of the brains of rats from the same group autopsied at 20 days or less after irradiation showed no recognizable morphologic changes.

exposed to 26,000 rad, suggesting that the brain tissue of the monkey is almost three times as radiosensitive as that of the rat to this dose. The brain of monkeys given 3000 rad, however, did not stain with fluorescein, suggesting that the dose-response curve for monkeys may have a steeper slope than that for the rat when plotted as shown in Fig. 4 and that the monkey may not show a greater radiosensitivity of brain tissue at lower radiation doses. The work of Hager and co-workers, using x radiation, indicates that the hamster brain is probably quite radiosensitive.[16]

In an attempt to rule out the possibility that the data collected in this study pertained specifically to the age of animal and the size of the aperture that was chosen originally, the influence of age and size of aperture were investigated to some extent. Rats 22 days old gave the same response as did rats 120 days old. There was no demonstrable difference in biologic effectiveness with aperture size ranging from 0.5 to 4 mm. This information is presented because it tends to emphasize the species differences that were observed and to permit broadening of interpretation. The findings are not intended to imply that age and size of lesion are not important factors in more extreme situations. Hicks has demonstrated that the adult nervous system is radioresistant and that the embryonic nervous system is radiosensitive.[17] Lindgren has stated that in clinical practice it is advisable to reduce the adult dose by 25% for treating brain lesions in 5-year-old children and by 50% in treating 2-year-old children.[11] Zeman et al. have demonstrated that in order to produce a lesion in the brain when the aperture is markedly narrowed (25 μ), an extremely high dose must be delivered to the tissue.[18] Differences in radiosensitivity between strains of mice have been demonstrated by Hicks and his associates,[19] and these investigators have shown differences in pathologic and physiologic effects when the intensity of the radiation is varied.[20]

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EFFECT OF A COMPLEXING AGENT AND MODE OF ADMINISTRATION ON THE DISTRIBUTION OF CURIUM-242 IN THE RAT

Marilyn H. Williams, Nylan Jeung, and Patricia W. Durbin

Some time ago we undertook a pilot experiment to determine the influence of a complexing agent and mode of administration on the distribution of curium. In view of the renewed interest in the biologic fate of the transuranic elements, it is appropriate to present the results of this preliminary work.

Highly charged cations, such as the lanthanides or actinides, are stable in acid solution (pH 2 or less). Colloidal aggregates tend to form as the pH is increased to make the solution suitable for animal administration.[1, 2] The tendency towards colloid formation is even greater at the pH of the body fluids in the presence of phosphate and protein. When injected intravenously, colloids formed either in solution or in the circulation are accumulated by the reticulo-endothelial system.[3] If specific data concerning distribution of a particular radioelement are desired, rather than those concerning distribution of a radioactively tagged colloidal particle, special pains must be taken to avoid colloid formation, either in the injection solution or following administration.[3, 4] Most of the lanthanides and actinides are absorbed poorly from the intestinal tract or when injected subcutaneously, intraperitoneally, or intramuscularly because of their great tendency to form insoluble compounds at body pH. One day after intramuscular injection of Pu, Am, or Cm (in isotonic saline at pH 5 to 6), the injection site retained respectively 77.1, 43.5, and 16% of the administered dose.[5-7] In all three cases the subsequent slow mobilization of radioactivity from the injection site greatly confused the interpretation of distribution data at postinjection times longer than 1 day. The intramuscular route, however, was chosen for most of the early survey work of element distribution, because colloid formation was suppressed, presumably by forcing the injected radioactivity to enter the circulation by diffusion into the capillary of lymphatic circulation a few atoms at a time. Subsequently it has been found that citrate would insure stability of neutral solutions of these highly insoluble elements, and that if high-specific-activity material was administered as a citrate complex, the citrate would be metabolized and the metal ion left to follow its own distribution pattern without significant colloid formation.[8, 9]

Before embarking on a large-scale investigation of the long-term retention of some of the elements with atomic numbers greater than 94 (transplutonic elements), it was necessary to decide upon a standard mode of administration. Intramuscular injection of the citrate complex was chosen for the following reasons: (a) a large number of animals could be injected accurately and quickly under very light ether anesthesia; (b) colloid formation in the circulation would largely be avoided; and (c) the addition of a complexing agent greatly facilitated absorption. These conclusions were drawn from previously published work[8, 9] and the results of this pilot study with curium.

Methods

The animals used were young adult female rats of the Sprague-Dawley strain, weighing 250 to 300 g. They were fed a pelleted stock diet and tap water ad libitum. Two solutions containing Cm^{242} at a concentration of 2.5 μC per ml were prepared. To one was added enough NaCl and NaOH to make the solution isotonic at pH 4. Sodium citrate was added to the other so that the final solution contained 15 mg/ml of sodium citrate at pH 6. Each rat was given 0.2 ml of one of these solutions either intramuscularly in the left hind leg or intravenously in the surgically exposed external jugular vein. Twelve rats constituted four groups of three rats each: intramuscular-saline (IM), intramuscular-citrate (IM-Cit), intravenous-saline (IV), and intravenous-citrate (IV-Cit). Each group was placed in a large metabolism cage for collection of excreta.

The animals were sacrificed with an overdose of chloroform 24 hr after injection. The kidneys, liver, spleen, gastrointestinal tract, and--in the case of the intramuscularly injected animals--both hind legs were dissected and weighed. These tissues, the carcass, and the urine and feces were dried and ashed in a muffle furnace. The soft tissue ash was removed from the residual skeleton by rinsing the bone ash with distilled water. The ashed tissues and excreta were dissolved in dilute HNO_3, and Cm^{242} was co-precipitated with LaF_3.[6]

Results and Discussion

The results of the 24-hour Cm^{242} tracer experiment are shown in Table I. Complexing greatly enhanced absorption from an intramuscular injection site. Only 2.8% remained unabsorbed at 24 hr in the IM-Cit group, contrasted with almost 20% in the injection site when no complexing agent was used (IM group).

The concentration of Cm^{242} in the spleen was low regardless of the mode of administration or the presence of a complexing agent. High splenic deposition is assumed usually to be associated with colloid formation. The data suggest that very little of the curium was colloidal, even when injected intravenously as $CmCl_3$. When Cm^{242} was given intravenously (with or without the complexing agent), the liver deposition was somewhat greater than when the injection was intramuscular. The difference, which was significant to the 2% confidence level according to the t-test,[10] could have been caused by the very small individual variations within each experimental group. The small difference between the skeletal deposition in the IM-Cit and IV-Cit groups was not significant ($P = 0.2$). In general, the tissue concentrations and the amount excreted by these two groups were very similar--the only difference of any significance being the liver. This difference may be more apparent than real, inasmuch as the livers of three rats injected intramuscularly with $CmCl_3$ in a previous experiment[7] contained on the average 59.6% of the absorbed dose at 24 hr.

The distribution of Cm^{242} in all four groups is remarkably similar to that observed in an earlier series;[7] the reproducibility of the 1-day data is highly encouraging.

Table I. Distribution of Cm^{242} in the rat 1 day after intramuscular or intravenous injection.[a]

	% absorbed dose				Administered dose			
	IM		IM-Citrate		IV		IV-Citrate	
	%	%/g	%	%/g	%	%/g	%	%/g
Spleen	.12	.20	.09	.15	.09	.15	.08	.12
G. I. tract	2.30	-	1.43	-	1.47	-	2.10	-
Liver	47.4	5.87	51.7	6.35	57.6	17.30	55.8	6.30
Kidney	4.14	2.25	3.59	1.90	3.41	2.02	3.37	1.74
Skeleton	28.5	1.20	27.8	1.16	22.2	.87	24.3	.99
Balance	9.16	-	8.48	-	8.75	-	8.31	-
Urine	7.99		6.54	-	5.77	-	5.38	-
Feces	.31		.39	-	.51		.57	-
S. D. liver	± .7		± .5		±1.1		±3.1	
S. D. skeleton	±1.2		±1.3		±2.0		±2.0	
Injection site	19.9		2.77	-				
Actual recovery	93.3		94.4	-	86.7		98.8	

[a]Each animal received 0.5 μC of Cm^{242}; citrate groups received the Cm^{242} complexed with 3 mg sodium citrate. Values are expressed either as per cent of absorbed dose (IM studies) or per cent administered dose (IV studies), and are corrected for deviation of recovery from 100%.

In the original experiments with Cm^{242}, the total excretion during the first 24 hr was approximately 4.5%;[7] total excretion in the current series was similar--about 6% of the absorbed dose. The earlier findings--65% of the excreted Cm^{242} in feces collected on the first day and 35% in the urine[7]--are probably in error. The urine drain in the old-style metabolism cages frequently became clogged with feces, allowing urine-borne radioactivity to be taken up by the fecal matter. This method of collection often led to large experimental errors. Since that time, the design of the metabolism cages has been improved, and the present findings, 90 to 95% of the excretion in the urine on the first day, are probably more reliable. The predominance of urinary elimination during the first day after injection has been observed repeatedly for all the lanthanides[11] and for actinium[12] and neptunium.[13]

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CONVERSION OF TESTOSTERONE TO ESTROGENS IN HUMAN BEINGS

Jorge A. Braun-Cantilo,* Gilles La Roche, and John H. Lawrence

As early as 1931 Steinach and Kun[1] suggested that the ovaries or the testes can produce either androgens or estrogens. This view was also supported by Lipschutz[2] and Hill.[3,4] In 1936 Steinach et al. demonstrated an increase in urinary estrogen-like material after the administration of androgens in normal and castrated rats[5] and normal human beings.[6] Several others confirmed these observations and extended them to other species.[7-12] By treating adrenalectomized and oophorectomized human beings with massive doses of testosterone, West, Damast, Sarro, and Pearson were able to observe an increase of urinary estrone and estradiol.[13] In this experiment, doses of 100 to 200 mg of unlabeled testosterone were used, and it is not possible to rule out a stimulation of independent pathways of estrogen formation.

In 1960, Breuer, Dardenne, and Nocke showed that daily doses of 60, 100, and 200 mg of 17 α-ethynyl-19-nortestosterone acetate were associated with the excretion of 17 α-ethynyl-17β-estradiol.[14] These findings indicate that human tissues may convert the A-ring of a 19-nor steroid to a benzene ring, which may serve to explain part of a suggested pathway[15] in the conversion of testosterone to estrogens (Fig. 1).

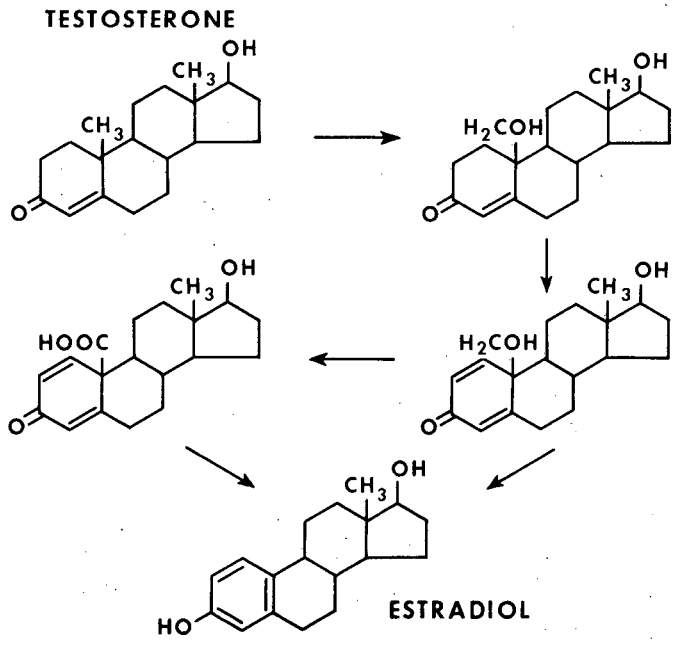

Fig. 1. Possible pathways in the formation of estradiol from testosterone; (taken from L. L. Engel, The Biosynthesis of Estrogens in Cancer 10, 711 (1957)).

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*Fellow, Argentine National Council of Research.

The conversion of testosterone-4-C^{14} to C^{14}-labeled estrogens has been demonstrated by means of tissue culture techniques.[16-20]

The only direct evidence for the in vivo conversion of labeled testosterone to labeled estrogens was demonstrated in the pregnant mare.[21] The work described in this paper furnishes evidence in support of a direct conversion of labeled testosterone to labeled estrogens in pituitary-irradiated human females.

Material and Methods

Subject 1

A 56-year-old female (E. B.) underwent a left radical mastectomy in February 1950 for breast cancer; pituitary irradiation was performed in June 1955 in an attempt to induce a remission of metastasis. At this time, she received high-energy proton irradiation to the pituitary, according to a procedure developed in this laboratory.[22-25] After irradiation, the patient was given replacement doses of adrenal and thyroid products. In order to prevent extensive protein catabolism, varying amounts of Nilevar (17-α-ethyl-17β-hydroxy-19-nor-Δ^4-androstene-3-one) and (or) testosterone accompanied the essential hormonal replacement. Shortly after the treatment, remission of cancer was observed. Four years later, a slight soreness was felt at the site of a sternal metastatic lesion, apparently concomitant with testosterone administration.

On October 13, 1959, she was injected intramuscularly with approximately 20 mg of testosterone-4-C^{14} (approx 16 μC)*, suspended in oil. The possibility of breakdown of the cyclopentanophenanthrene nucleus followed by the loss of radioactivity in the form of expired CO_2 was ruled out by counting the air for a period of 6 hr following injection.

Urine was collected at the following intervals: 0 to 2 hr (sample A), 2 to 6 hr (B), 6 to 12 hr (C), 12 to 24 hr (D), and 24 to 48 hr (E). A control sample of urine had been collected during the 24 hr prior to the injection (sample X).

Subject 2

A 46-year-old woman (I. W.) had a right radical mastectomy in January 1955 and an oophorectomy in October 1957. The patient had bone metastasis and received pituitary irradiation in January 1958. Replacement therapy including adrenal and thyroid products was administered.

On January 19, 1960, she received 7.62 mg of testosterone-4-C^{14} (15.48 μC) by intravenous infusion. The testosterone was dissolved in pyrogen-free absolute ethanol (12 ml), and this solution was made up to 500 ml with normal saline. The patient received 387 ml of preparation, and the remainder was kept for use as standards.

The urine samples were collected at the same intervals as for Subject 1. After the second day, the samples were collected in 24-hr fractions until the tenth day. A final

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*These values are approximate, since the preparation was later found to be nonhomogeneous.

24-hr sample was obtained on the 17th day. A urine sample collected on the day prior to infusion served as the control. Feces were also collected in 24-hr samples for the 10-day period following infusion.

Analysis

The urine samples were measured and kept frozen until processed. Aliquots of 40 ml of each sample were treated as indicated in Fig. 2. The difference between the purified extracts No. 2 and No. 3 is due to the way in which they were washed. Extract 2 (Method 1) includes the neutral but not the phenolic (estrogens) steroids; Extract 3 (Method 2), on the other hand, contains the estrogens. That is to say, in the extract obtained by Method 1, the washing would have removed the estrogens, whereas in Method 2, the washings would have removed most of the phenolic acids, but not the estrogens.

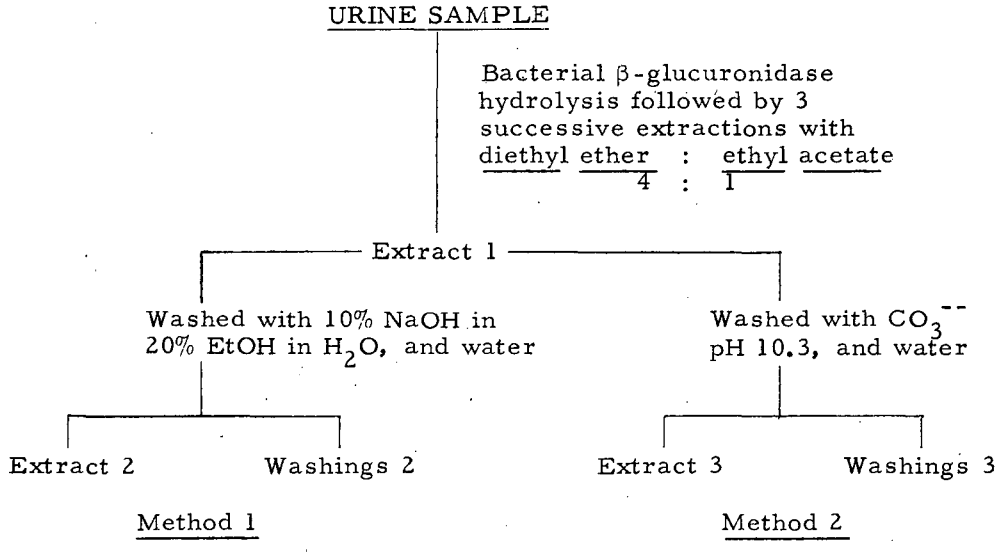

Fig. 2. Flow sheet for the isolation of urinary steroids.

The 17-ketosteroids and 20, 21-ketosteroids were measured chemically in both extracts[26] (Tables I and II). One-tenth of each extract was put into a vial to count in a liquid scintillator. One-tenth of the same extract was put at the origin of a paper chromatogram for fractionation.

The extracts were concentrated to a small volume in a special evaporator illustrated in Fig. 3. For large volumes, a circulating evaporator was used, and in every instance the temperature was maintained below 45°C.

The feces were blended and lyophilized, and the weighed powder was kept at -20°C until processed as indicated in Fig. 4. The feces were extracted in a modified Soxhlet apparatus (Fig. 5). Extracts 1 and 2 of the feces, containing the lipid extractable material, were combined and concentrated to 100 ml, 1 ml of which was put into a vial for counting. Extract 3, containing conjugates (β-glucuronides), was concentrated to 50 ml, and 1 ml was counted.

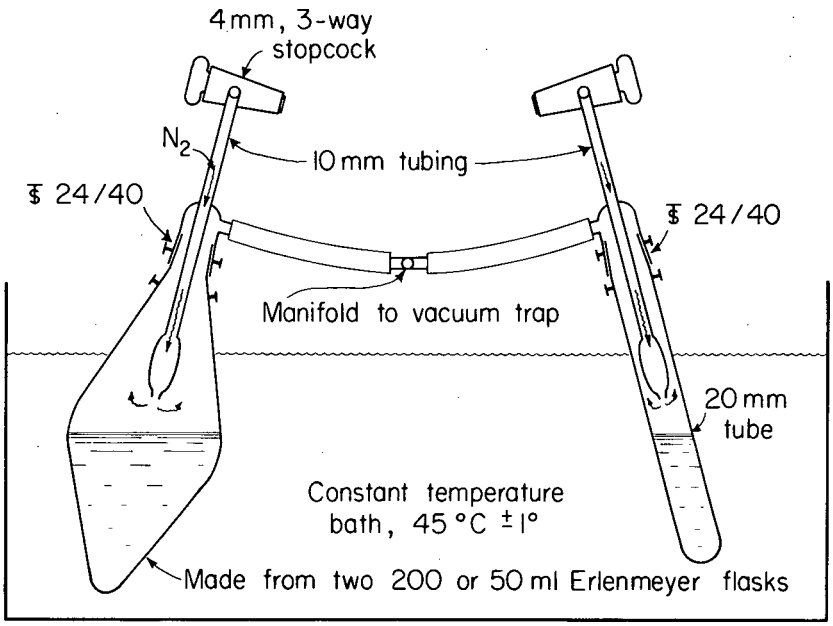

Fig. 3. Evaporator used in concentrating extracts.

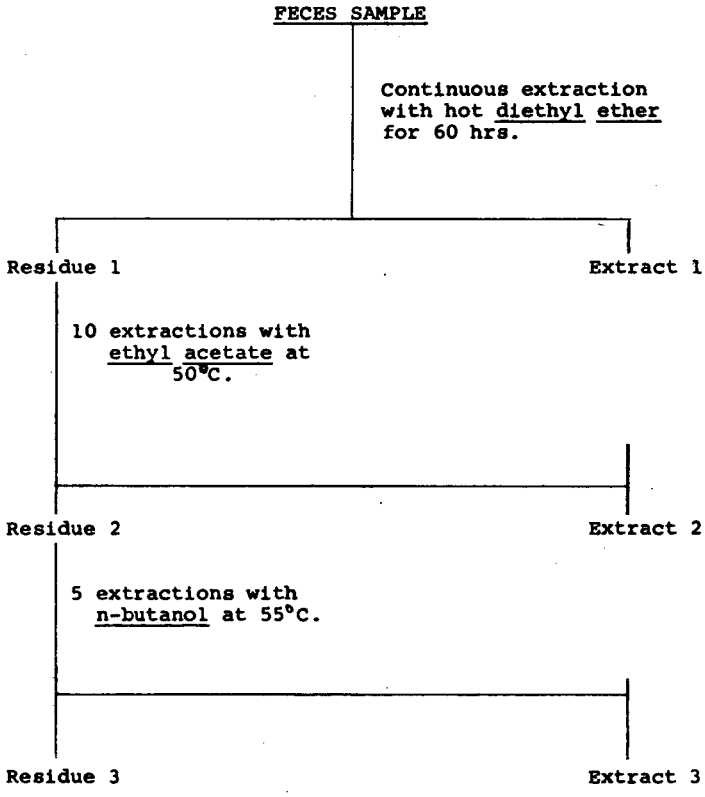

Fig. 4. Flow sheet for the isolation of fecal steroids.

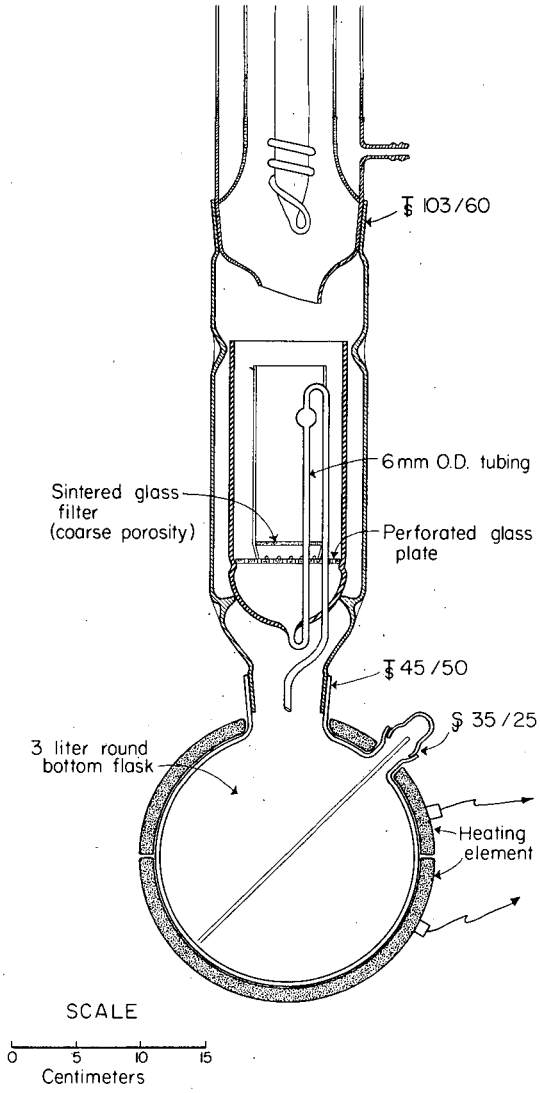

Fig. 5. Modified Soxhlet apparatus used to extract the feces.

Paper chromatography of the urine extracts was done on 1×40-cm strips of Whatman paper No. 20. In some cases, this paper had been washed previously with methanol, using the apparatus shown in Fig. 6. The solvent mixtures used in these descending chromatograms consisted of benzene:methanol:water (11:7:2) and hexane:methanol (11:5:10). The chromatograms were developed in the dark for 12 hr with the first solvent mixture and for 30 hr with the second. After the chromatograms were dried, the phenolic steroids could be visualized as blue spots by the Folin-Ciocalteau test. The androgens were visualized by spraying with a 1:1 mixture of 15% KOH in methanol and 1% m-dinitrobenzene, and heated at 70 to 100°C until spots appeared (Fig. 7). These chromatograms were subsequently scanned and autographed by contact. The distribution of the radioactivity was studied in relation to the positions of standards.

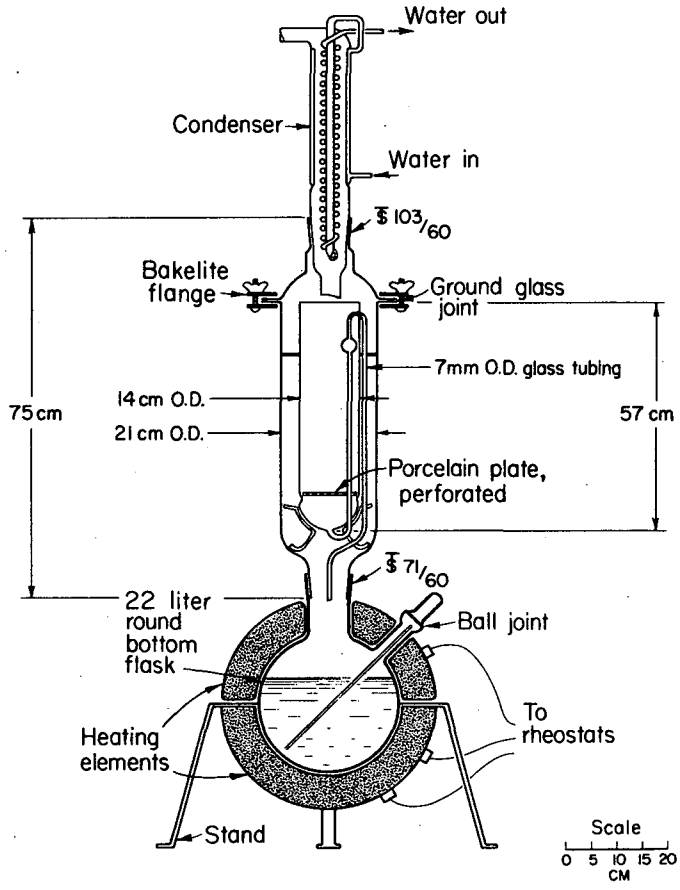

Fig. 6. Apparatus used to wash the chromatography paper.

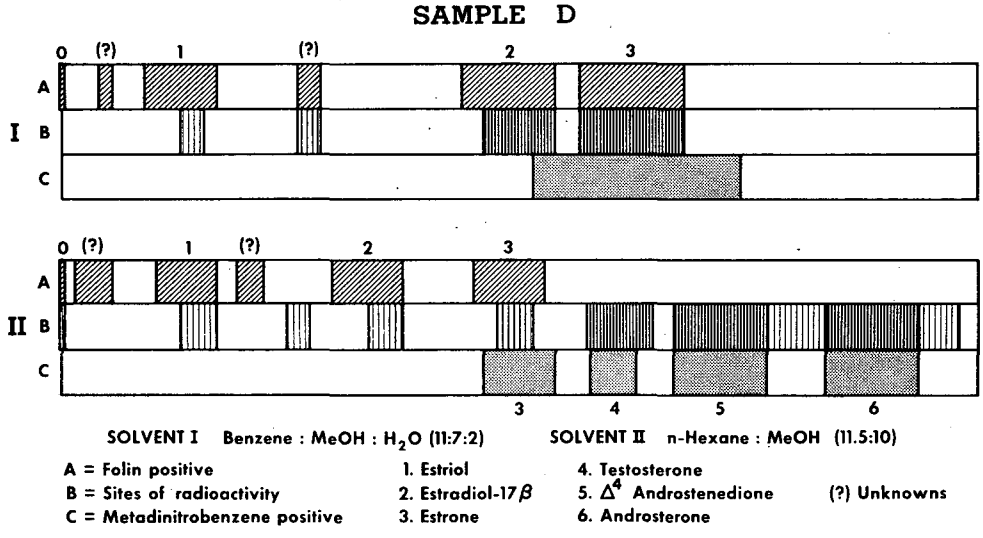

Fig. 7. Composite pictures of paper chromatograms of an extract run in two different solvents and developed by three different means.

Radioactivity was measured with a Tri-Carb liquid scintillator. In order to calculate the absolute efficiency with which each sample was counted, internal-standard counting techniques were used. Labeled toluene was utilized for this purpose. The phosphors used were PPO, 3 g per liter, and POPOP, 0.05 g per liter in toluene.

Residue 1, washings 2 and 3 (Fig. 2) were lyophilized; the powdered material was weighed, and an aliquot was transferred to a counting vial. The powdered material to be used for counting was suspended in toluene with 25 g/l Thyxcin, and 3 g/l PPO as a phosphor.

Results

Table I shows the levels of total urinary 17-keto steroids and 20, 21-ketol steroids in the various extracts obtained from Subject 1. The table also includes the total urinary volumes for each of the collection periods. The 17-keto levels observed in both extracts appear to be in somewhat better agreement than corresponding values for the 20, 21-ketols. Similarly, these levels observed in Subject 2 (Table II) seem to confirm this observation even though it becomes apparent that the 17-keto values are higher in Extract 3 than in Extract 2.

Table I. Chemical determinations of 17-keto steroids and 20, 21-ketol steroids in Subject 1.

Sample		Volume	17-keto Steroids*		20,21-ketol Steroids**	
Day	hrs.	ml.	Extract 2	Extract 3	Extract 2	Extract 3
	0-2	415	0.3	0.3	1.2	2.3
	2-6	156	1.4	1.2	1.5	2.4
	6-12	478	0.9	1.6	2.0	3.5
	12-24	1176	2.1	1.1	3.3	6.5
1st		2225	4.7	4.2	8.0	14.7
2nd		2300	2.0	2.3	9.3	19.2
X		2165	0.3	0.6	6.1	12.1

* Expressed as mg. equivalent of Epiandrosterone (Dehydro) per total volume.

** Expressed as mg. equivalent of Hydrocortisone per total volume.

Both of these two series of extracts in Subject 2 were counted and plotted (Fig. 8) in order to visualize any difference in the counting rate that may reflect the presence of labeled phenolic estrogens. After the first day's collection it appears that indeed Extract 3, which would contain the phenolic estrogens, has higher radioactivity than Extract 2, which would be devoid of these products. Even though this difference is noticeable only in extracts from days 2, 3, 4, 5, 6, and 7, it would seem that the ephemeral appearance of phenolic estrogens as urinary metabolites would be compatible with the single infusion of a precursor.

Table II. Chemical determinations of 17-keto steroids and 20, 21-ketols in Subject 2.

Sample		Volume	17-keto Steroids*		20,21-ketol Steroids**	
Day	hrs.	ml.	Extract 2	Extract 3	Extract 2	Extract 3
	0-2	540	1.6	1.4	0.9	1.9
	2-6	150	1.1	0.8	2.3	2.3
	6-12	333	0.5	0.4	2.0	2.8
	12-24	1218	1.3	1.3	5.2	9.5
1st		2241	4.5	3.9	10.4	16.5
2nd		1341	1.3	1.1	7.4	11.6
3rd		2560	0.8	1.4	6.6	19.2
4th		1865	0.4	0.6	3.1	14.4
5th		1415	0.4	1.4	3.7	20.4
6th		1400	0.4	1.0	4.2	18.3
7th		2030	0.5	1.5	4.6	20.5
8th		1830	0.7	1.5	6.2	21.2
9th		1530	0.4	1.1	5.0	19.1
10th		1700	0.8	1.1	9.1	24.7
17th		1020	1.4	0.9	7.5	14.2
X		2082	1.2	1.2	10.3	22.1

* Expressed as mg. equivalent of Dehydroepiandrosterone per total volume.
** Expressed as mg. equivalent of Hydrocortisone per total volume.

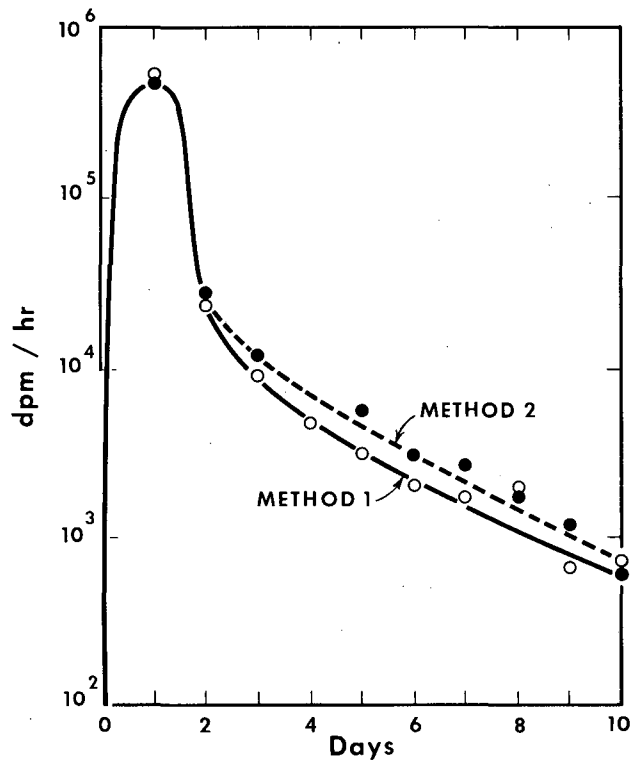

Fig. 8. A semilogarithmic plot of the radioactivity recovered from the urine by two different methods.

The per cent recovery of radioactivity was computed only for Subject 2, since the labeled testosterone preparation administered to Subject 1 did not appear homogenous. Subject 2 was given a total number of counts estimated at 3.4×10^7 dpm. The total excreted in the urine was 2.34×10^7 dpm, accounting for 68.9% of the injected dose.* After β-glucuronidase hydrolysis, approximately 57.5% of the radioactivity became estractable, accounting for 1.35×10^7 dpm, or 38.3% of the injected amount. The extractable counts from the feces amounted to 7.7% of the injected dose, or 2.6×10^6 dpm (Table III). Thus, 46% of the injected radioactivity could be accounted for in the various extracts.

Table III. Radioactivity recovered from the feces in the various extracts.

Sample DAY	Extracts 1 + 2 DPM	Extract 3 DPM	Total DPM
1st	848 500	25 200	873 700
2nd	34 950	1 780	36 730
3rd	159 000	30 900	189 900
4th	280 500	63 500	344 000
5th	151 500	37 300	188 800
6th	419 000	72 500	491 500
7th	153 500	67 500	221 000
8th	72 700	15 550	88 250
9th	42 350	36 800	79 150
10th	46 500	52 700	99 200
Total	2 208 500	403 730	2 612 230

With the exclusion of the first day, a slightly higher degree of radioactivity was observed in the extracts that should contain estrogens than in those devoid of them (Figs. 2 and 8). This difference seems to persist for at least a week following the time of infusion (Fig. 8). Figure 7 represents the results of androgenic and estrogenic steroid chromatographic fractionation of urine sample of the last 12-hr period of the first day. These diagrams represent composite pictures of chromatograms developed with two solvent systems, in which the steroid concentration in bands was visualized by means of various techniques (Fig. 7). The chromatograms from system I stained by Folin-Ciocalteau (Fig. 7, IA) yielded six separate bands, including the one for origin. On the

*A method is presently being developed to measure the total radioactivity in the feces.[27]

basis of these and by comparison with standards, estriol was clearly separated from estradiol-17 and estrone, whereas only a partial separation could be visualized between the latter two. As indicated by the autograph in Fig. 7, IB, all three of the estrogens (estriol, estradiol-17β, and estrone) reveal significant levels of radioactivity. In addition, an unknown (?) Folin-C positive band exhibits a degree of radioactivity. When the chromatogram is stained with metadinitrobenzene, only one large band can be observed with a midpoint at the level of estrone as stained by Folin-C (Fig. 7, IC). From the evidence gathered so far, testosterone and its androgenic metabolites appear run off the paper 2 with this system.

In the second solvent system (Fig. 7, II) it appears that we obtain a better fractionation of estradiol-17β and estrone, as evidenced by Folin-C-staining (Fig. 7, IIA). As with the previous solvent system, there are still two unknowns, neither of which coincides with autographic bands in this instance (Fig. 7, IIB). Again it is possible to observe radioactivity at the levels of all the estrogens (compare A and B), testosterone, and its various metabolites, as identified by the subjacent strip stained with metadinitrobenzene (Fig. 7, IIC). Two additional radioactive bands do not appear to coincide with either a positive Folin-C or metadinitrobenzene band.

Discussion

The small urinary volumes that are noticeable in the 2- to 6-hr samples (Tables I and II) can be attributed to the water-retaining effect of adrenal products that were administered just as this collection was started.

The difference between the 20, 21-ketol values obtained from extracts by Method 1 and Method 2 would suggest that the higher values are due to nonspecific reducing products that are removed in Method 1.

In our discussion of Subject 2 it should be pointed out that the estimated recovery based on radioactivity of the extracts does not reflect the excreted labeled products in toto. It is only when aliquots of feces have been counted and the sums added to urine counts that it will be possible to establish the amount of the injected labeled testosterone that has been excreted. In addition, from the counts present in the last extracts of feces (Table III), it is apparent that the collection should be extended for a longer time in order to obtain the total radioactivity excreted by this route. Further, it would appear plausible to expect a certain amount of labeled products to be excreted through the skin.[28]

Assuming that the radioactivity in the feces represents approximately half of the radioactivity present in this fraction, as shown in urine, and considering that an important amount of radioactivity may still be excreted through the feces even after 10 days, it would appear likely that 85% of the injected radioactivity could be recovered from urine and feces within 20 days following injection. The remaining 15% unaccounted for may be (a) included in fecal and urinary excretions beyond 10 days after injection, (b) excreted through sweat, as several steroids are known to be, or (c) lost in the collection of the

first five urine samples, for instance. These assumptions would suggest that in a well-controlled account of the radioactivity, a negligible amount of testosterone administered by a single infusion would remain in the body after a short period of time, contrary to general belief. At this time no effort has been made to sample sweat and measure its radioactivity, but plans have been made to explore this avenue of excretion in the future.

Several authors have presented evidence indicating the absence of estrogen-like material after the injection of testosterone,[29-31] but it would appear that most investigators favor the possibility of this conversion, even though in all but one case[21] they do not present definite indications of it. In their work in the pregnant mare Heard et al.[21] have shown a 1% transformation of testosterone to estrogens. In this case, the production of estrogens should undoubtedly be at a maximum, and it is expected that if there were transformation, it would probably be also at a maximum. Even so, the authors have not been able to detect more than 1% transformation. In our study the recovery was made from an oophorectomized woman with an impairment of pituitary function, and it is expected that this transformation would be at a minimum, even though there is evidence for the conversion of testosterone to androsterone, Δ^4-androstenedione, and etiocholanolone (Fig. 7).

From the chromatographic evidence thus obtained it is possible to see that the largest portion of radioactivity is indeed associated with the androgens (Fig. 7, II). On the other hand, it is also quite clear that a certain amount of radioactivity remains associated with at least three estrogens (estrone, estradiol-17β, and estriol) (Fig. 7). At present, this finding is considered as a further indication of the conversion of testosterone to estrogens.

Elution of the chromatographically separated estrogens containing radioactivity and the constancy of the specific activity through several recrystallization would confirm the above findings.

Acknowledgments

We want to extend our thanks to Drs. Richard M. Lemmon, Cyril Ponnamperuma, William G. Dauben, Harold Werbin, William Siri, and Gerard Milhaud. The assistance of Irving Ford, Mrs. Ghislaine Herman, Miss Mary Novitski, and Mrs. Jennie Mae London is gratefully acknowledged.

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AUTORADIOGRAPHIC OBSERVATIONS OF PLASMA CELL FORMATION*

John C. Schooley

It is well known that lymphoid tissue is involved directly in antibody formation, but the role of the individual macrophage, lymphocyte, eosinophil, or other cell types in initiating antibody formation has not yet been established. There is some evidence that cells of the plasmocytic series contain, release, and probably synthesize antibody.[1-5] High-resolution autoradiography, following the administration of tritiated thymidine to mice, has been used to study plasmocytopoiesis in the regional lymph nodes of the mice after multiple injections of antigen, and the findings are presented in this paper. Some preliminary observations of the incorporation of tritiated cytidine and leucine into cells of the plasmocytic series are also included.

Materials and Methods

Male Swiss mice, weighing about 26 g, were used in all experiments. The mice were injected subcutaneously in the right hind leg with 0.05 ml of a 1:1:1 mixture of dog serum, Freund's adjuvant, and Gey's solution on alternate days for a total of three injections. The presence of antibody against dog serum in such animals was confirmed. One day after the last injection of antigen, one group of animals was injected intraperitoneally with 1 μC per g body weight of H^3-thymidine (360 mC per mM); another group received 1 μC per g body weight of H^3-cytidine (360 mC per mM); and the last group, 5 μC per g body weight of DL-leucine-4, 5, H^3-hydrochloride (3000 mC per mM). Mice that had been given H^3-thymidine were sacrificed at 1, 4, 8, 12, 24, 30, 36, 48, or 60 hours, or 3, 4, 5, 6, 7, 10, 14, or 49 days after injection. Three animals were sacrificed at 1 hour, three at 8, and two at each of the later intervals. H^3-cytidine-treated mice were sacrificed 0.5, 1, 4, 8, 12, 18, 24, 30, 36, 48, or 72 hours after injection: two animals at 1 hour and one animal at each of the other times. H^3-leucine-treated mice were sacrificed at 0.5, 1, 5, 8, 24, 48, or 72 hours after injection: two animals at 1 hour and 1 animal after each of the other intervals. At the time of sacrifice, smears were made of blood, thoracic duct lymph, and bone marrow as well as of cell suspensions of thymus, spleen, and mesenteric, cervical, and right (stimulated) and left inguinal lymph nodes. Cell suspensions were made by teasing apart tissue samples, in several drops of serum, with needles. Also sections of these tissues were prepared.

Smears were fixed overnight in either absolute methyl or ethyl alcohol. Sections were fixed in neutral buffered formalin and embedded in paraffin. Sections were cut at 4 μ.

Autoradiographs were made of all smears and sections using Kodak Ltd. (England) AR-10 stripping film. They were exposed for 30 days in the cold, then developed and fixed.

*Accepted for publication in the Journal of Immunology.

Smears were stained with either Giemsa or pyronine and hematoxylin; sections with hematoxylin-eosin or eosin-azure II.

The autoradiographs were examined, and the percentage of labeling in the different classes of plasmocytic cells as well as the total number of grains over each labeled cell was determined for mice at each time of sacrifice after injection.

Results and Discussion

Morphologic and Cytologic Observations

The stimulated right inguinal lymph node underwent hypertrophy, and by the time the series of antigen injections was finished, the node was about four to five times the size of the contralateral node. At this time an enormous increase in large, approximately round cells with quite basophilic cytoplasm was observed in sections, particularly those near the arterioles of the medullary cords, although diffuse nests of these cells were seen throughout the node. About 12% of the cells found in smears of the hypertrophied nodes were classified in the plasmocytic series. The sinuses were generally dilated and filled with cells that appeared to be primarily medium and small lymphocytes. Except for the fact that no substantial increase in the number of germinal centers was observed, the histologic changes were similar to those described by other workers who observed regional lymph nodes of rabbits[1,6] and guinea pigs[7] after antigenic stimulation with bacteria or bacterial products.

For scoring purposes, plasmocytic cells were classified into five categories representing differences in maturation (Fig. 1), as follows.

(a) Early plasmoblast. Large round cells with basophilic or pyroninophilic cytoplasm having a leptochromatic nucleus with a diameter greater than 10 μ. One to several nucleoli were revealed by pyronine staining, but were less distinct after Giemsa staining. These cells presumably correspond to the transitional cells described by Fagraeus[1] and the "activated reticulum cell" of Marshall and White.[8]

(b) Late plasmoblast. Similar to the preceding cell but having a nuclear diameter of 9 to 10 μ and rather indistinct nucleoli. This cell has been termed a plasmoblast[8] and immature plasma cell.[9] If the cytoplasm of this cell were less basophilic it would be indistinguishable from the large lymphocytes that appear in germinal centers. A small number of such cells is always found in the thoracic duct lymph of animals that have not been exposed intentionally to antigens, and it has been shown that under appropriate conditions these cells can become typical plasma cells.[10]

(c) Proplasmocyte. An oval cell having a round or slightly oval eccentrically located nucleus with a diameter of 8 to 10 μ. The chromatin pattern is coarser than that in the previously mentioned cells.

(d) Early and late plasmocytes. These are the most mature plasma cells. The early plasmocyte has an eccentrically located nucleus with a diameter of 7 μ or less, a perinuclear clear area, and abundant basophilic quite granular cytoplasm. The nucleus is identical

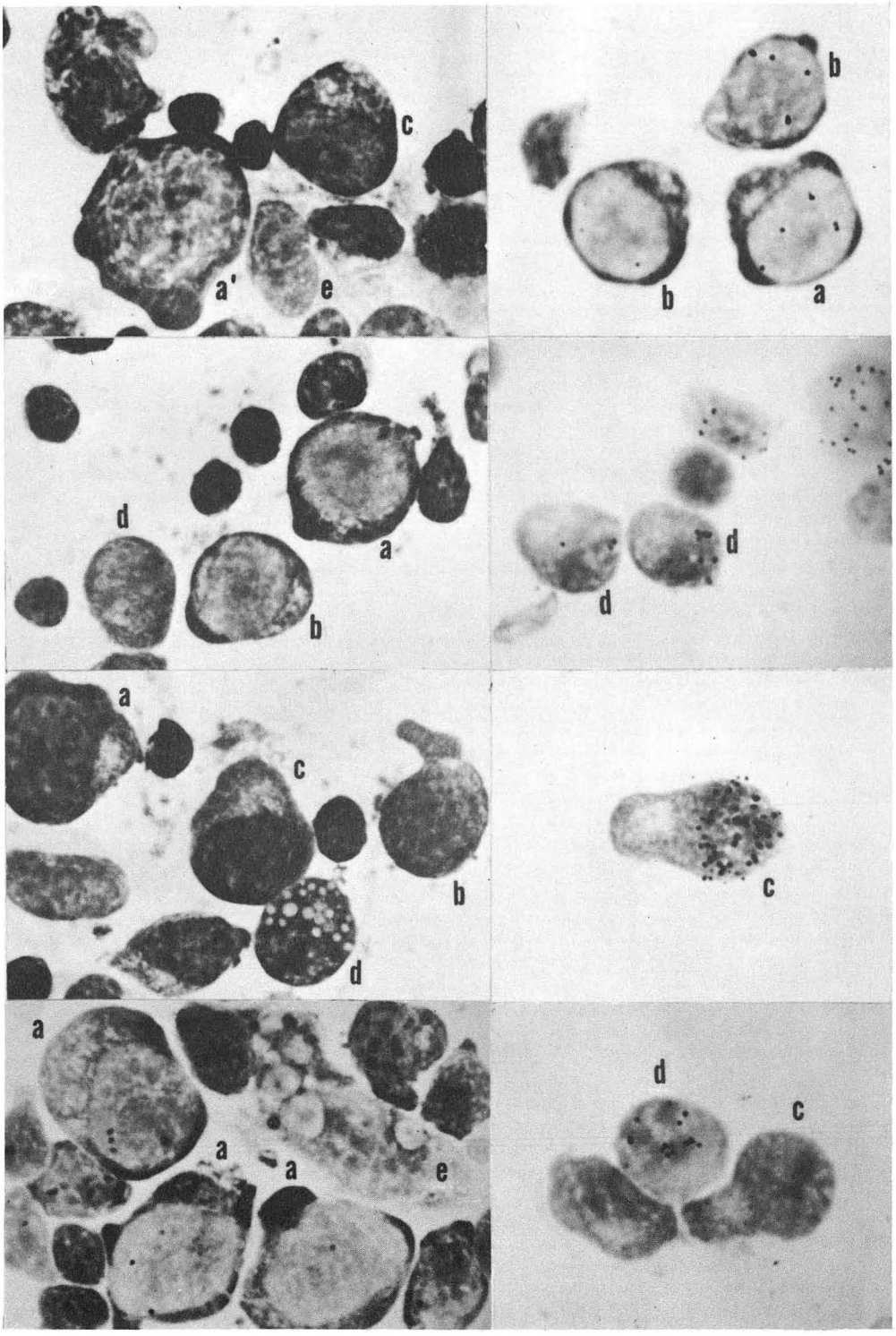

Fig. 1. Labeled and unlabeled plasmocytic cells found in smears of the multiply stimulated lymph nodes of mice. (a) Early plasmoblast; (a') prophase of early plasmoblast; (b) late plasmoblast; (c) proplasmocyte; (d) early and late plasmocyte; (e) reticulum cells. Mag, X 2200.

to that seen in small lymphocytes. The late plasmocyte is the same as the early one except that the nuclear chromatin has the typical cartwheel arrangement of a Marshalko plasma cell. Late plasmocytes were seldom seen, and early and late plasmocytes have generally been grouped together in scoring. Vacuoles and multinucleate forms were seen commonly in these mature cells. None of the plasmocytic cells had the capacity to phagocytize colloidal particles.

Relatively few large lymphocytes were seen in stimulated nodes. These cells have nuclear diameters of 10 μ or greater and light blue cytoplasm. They are the prevalent large-cell type seen in normal nodes. Their presence might suggest relatively inactive lymphocytopoiesis; but it was observed that the lymph sinuses were dilated enormously and filled with lymphocytes of various sizes. Ehrich and Harris have observed a decided increase in the output of lymphocytes,[11] and probably plasmoblasts and plasma cells,[12] in the efferent lymph draining a stimulated node, while the lymphocyte content of the afferent lymph was not greatly increased. This suggests that the increase in the lymphocytes in the node is the result of proliferation rather than migration into the stimulated node of lymphocytes that have been produced elsewhere.

Large numbers of small round cells were seen in smears of the stimulated nodes. The cytoplasm of these cells was much more basophilic and pyroninophilic than that of the small lymphocytes of unstimulated nodes. Electron microscopic studies of these cells reveal that they have a more lamellar endoplasmic reticulum than the similar-sized cells of nonstimulated nodes or thoracic duct lymph.[13] Keohane and Metcalf have shown that the cytoplasmic refractive index of the small lymphocytes isolated from various lymphoid tissues after antigenic stimulation increases,[14] and they propose that this increase is due to an accumulation of some unknown protein. The role of these cells in antibody production, whether they contain antibody or antigen and whether they are plasmocytic or lymphocytic, can certainly not be determined with the available information. The data are consistent with the suggestion of Wissler et al.[15] that both the mature plasma cell and this small round cell, which resembles a small lymphocyte, are derived from the divisions of a common more immature cell present in the stimulated node.

Autoradiographic Observations, H^3-thymidine

Basic premises concerning the use of H^3-thymidine and interpretation of the results have been discussed by various authors.[16-18] In order to obtain a more random sample of the total plasmocytic population, better cytologic detail of individual cells, and better quantitation of the number of grains over each labeled cell, all the present data have been obtained from observations of smears. Autoradiographs of sections were studied only to confirm qualitatively the information obtained from smears. Approximately 200 cells were counted in each of the separate categories of plasmocytic cells for each animal at each time. In determining the percentage of labeled cells, only cells with a count of at least three grains above representative background areas were considered to be labeled. The temporal pattern for the per cent labeling of plasmocytic cells is shown in Fig. 2. The

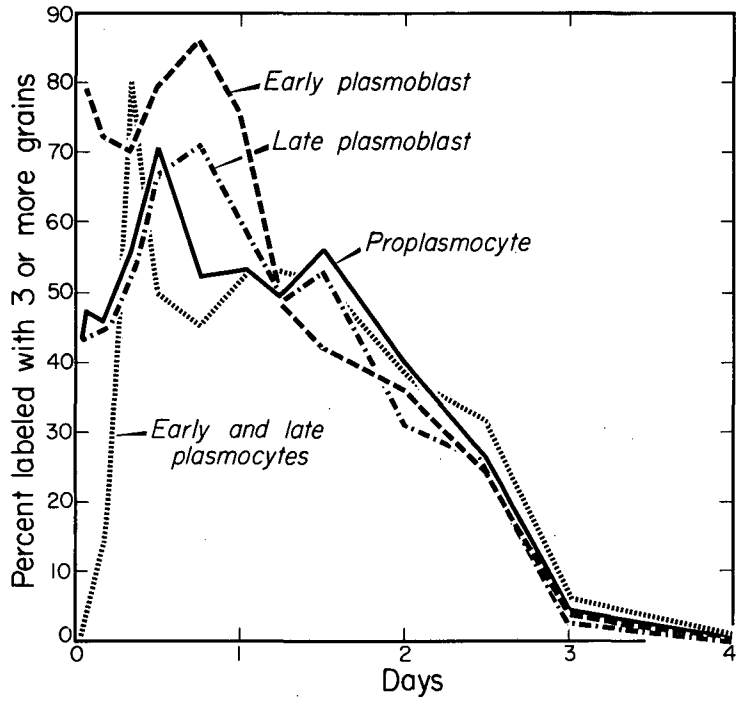

Fig. 2. Labeling of plasmocytic cells as a function of time following a single injection of H^3-thymidine.

values obtained for all animals after any one time interval were averaged. The rapid disappearance of labeled cells is striking. It can occur only through one of three processes--cell death, dilution of the label through successive division, or migration of labeled cells out of the node population. Migration of labeled plasmocytic cells from the stimulated node undoubtedly occurs, but the extent of this migration and its relative effect on the temporal pattern of labeling cannot be quantitated in these experiments. By 1 hr, 80% of the early plasmoblasts and 45% of the late plasmoblasts and proplasmocytes were labeled, whereas no labeled early and late plasmocytes were found. Presumably the labeling at this time has come from the endogenous synthesis of DNA and not from the division of precursor cells. If it is accepted that this endogenous synthesis is presumptive of future cell division, it appears that the most mature plasma cells are nondividing cells. A very rapid increase in the percentage of labeled early and late plasmocytes occured, and by 8 hr 80% of these cells were labeled. Presumably, therefore, the mean life within the node of the identifiable mature plasma cell is of the order of 8 to 12 hr. Figure 3 is presented to show the grain count distribution over labeled early plasmoblasts 1 hr after a single injection of H^3-thymidine. The number of grains observed over a cell at 1 hr depends on various factors such as the availability time of H^3-thymidine, the size of the precursor pool, and the extent and rate of DNA synthesis in the cell during the availability time of the immediate precursor. The general shape of this curve remained unchanged at later

times as long as sufficient numbers of labeled cells could be counted, an indication that division occurred randomly with regard to grain count. Figure 4 shows the temporal

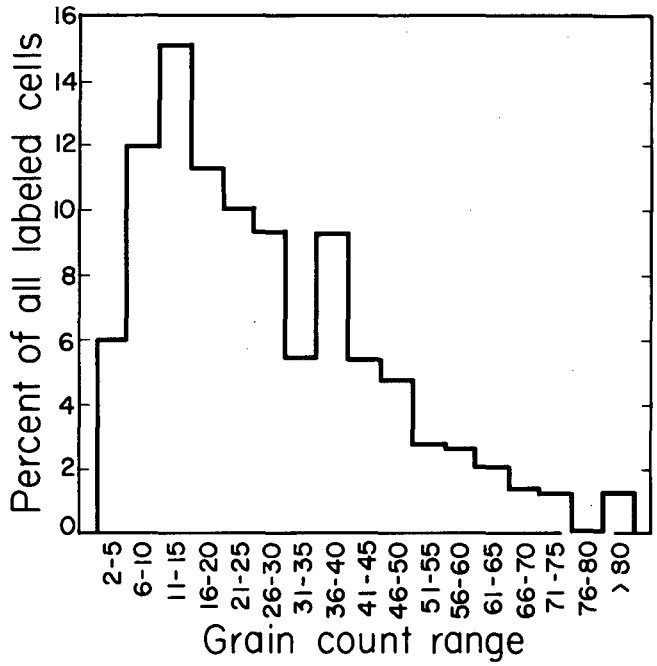

Fig. 3. Grain-count distribution of early plasmoblasts 1 hr after injection of H^3-thymidine.

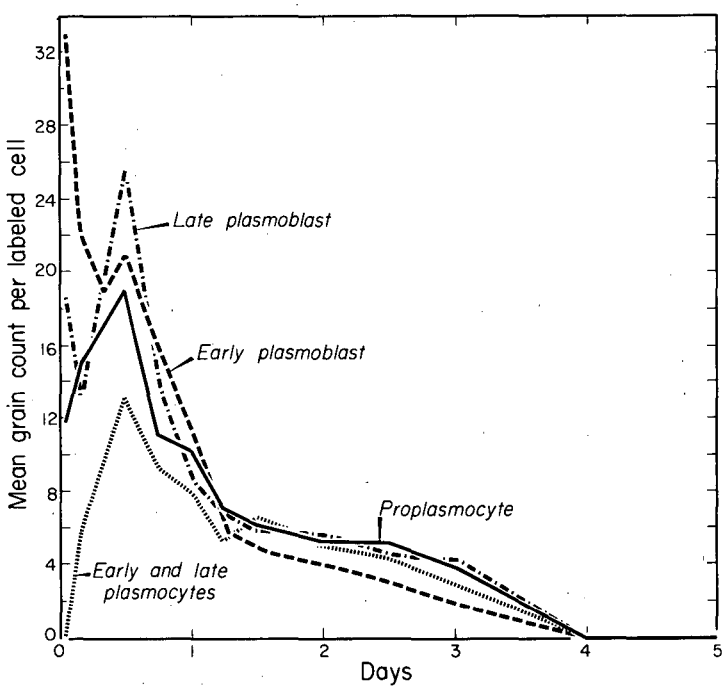

Fig. 4. Mean grain count of labeled plasmocytic cells as a function of time following a single injection of H^3-thymidine.

changes in the mean grain counts of all labeled plasmocytic cells. The mean grain count of late plasmoblasts is initially high, then decreases, and finally increases again at 12 hr. At the time of this second increase the mean grain of proplasmocytes and early and late plasmocytes reaches a maximum. The rapid decrease in the mean grain count of the early plasmoblasts seen in Fig. 4 has been plotted logarithmically as a function of time in Fig. 5.

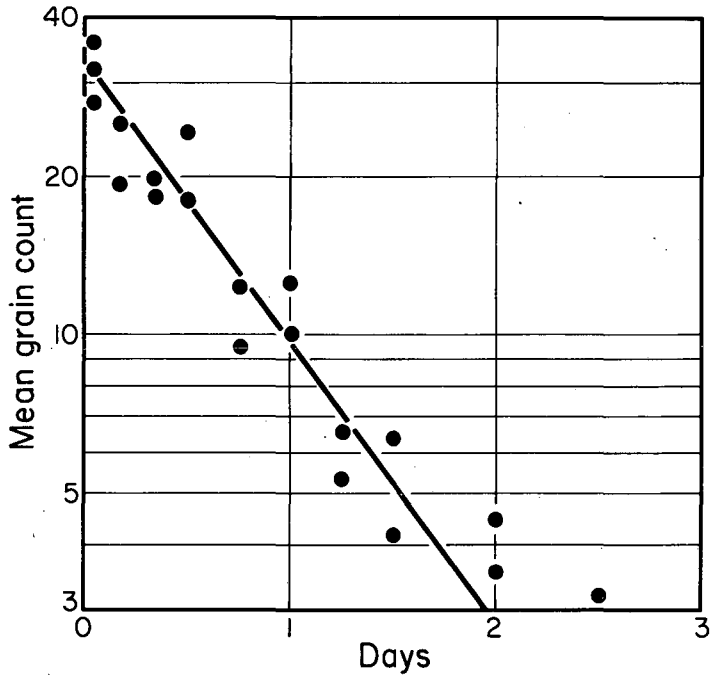

Fig. 5. Mean grain count of labeled early plasmoblasts plotted logarithmically as a function of time.

Each point represents values for the stimulated node of one animal. The time required for a half-reduction in the grain count indicates that the maximum generation time for early plasmoblasts is 12 to 14 hr. This is similar to values calculated in a similar fashion for large lymphocytes in vivo[16] and in vitro.[4] The facts that 80% of the early plasmoblasts are labeled at 1 hr and that anaphase figures of cells, presumed to be plasmoblasts, are observed at the same time suggest that the synthesis of DNA occurs during most of interphase. The gap between the completion of mitosis and the beginning of DNA synthesis and the gap between the end of DNA synthesis and the beginning of the next mitosis must be very short. The proliferative capacity of the plasmocytic series is further indicated by the observation that by 3 to 4 days after injection, H^3-thymidine-labeled cells cannot be detected in the node.

Initially, the small round cells discussed previously were unlabeled, but they increased to about 1 to 2% labeling by 1 day and then gradually decreased and were not detected after the fifth day. This is quite similar to the pattern reported previously for the small lymphocytes of nonstimulated mice,[16] except that the percentage of cells labeled

is slightly lower. Considering that these cells comprise at least 80% of the cells in smears and that extensive migration of these cells from the node is occurring, it is evident that extensive lymphocytopoiesis occurs in the stimulated lymph node.

Only rarely were phagocytic or nonphagocytic reticulum cells found labeled in sections of the stimulated nodes 1 hr after the injection of H^3-thymidine. It appears, therefore, that proliferation and subsequent differentiation of reticulum cells into plasmoblasts is not occurring in these multiply stimulated nodes, but such a reaction may well have occurred during the initial exposure of the lymph node to antigen. Interestingly, many phagocytic cells contained labeled nuclear debris at this time. Whether the labeled thymidine in this debris would be utilized during future synthesis of DNA by these cells could not be determined in the experiments presented here.

The flow of labeling from one class of plasmocytic cell to another indicates that the cytologic criteria used for separating these cells into different stages of maturation are reasonable.

H^3-leucine

One hour after the injection of H^3-leucine, every early and late plasmoblast and proplasmocyte was labeled, whereas only about 50% of the early and late plasmocytes was labeled. At 5 hr all plasmocytic cells were labeled and they remained labeled during the course of the experiment, although there were definite changes in the degree of labeling in each class of cell. The temporal changes in the mean grain count are seen in Fig. 6. If, during fixation, only free H^3-leucine is removed from the cells, it seems reasonable

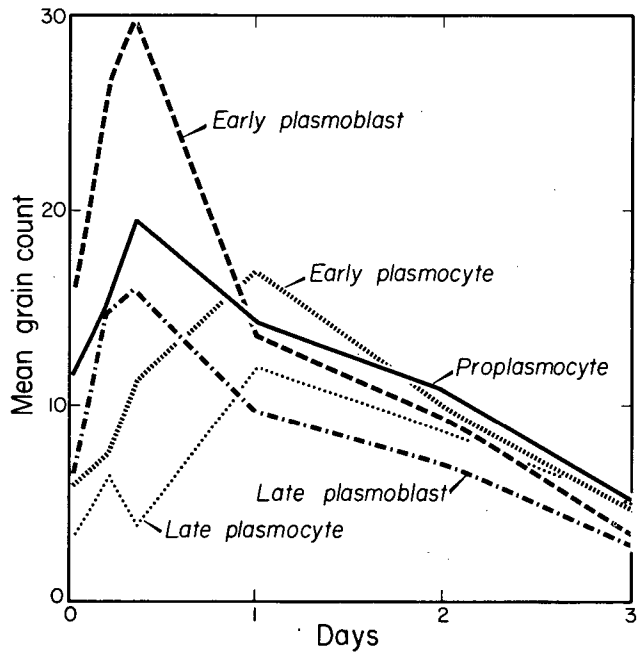

Fig. 6. Mean grain count of labeled plasmocytic cells as a function of time following a single injection of H^3-leucine.

to conclude that the number of grains over a labeled cell is proportional to the amount of amino acid incorporated into the protein and polypeptides synthesized during the time that the labeled leucine was available. The period of availability, the size of the precursor pool, and the extent of recycling of labeled leucine are unknown. The amino acid is undoubtedly being incorporated into cytoplasmic, nuclear, and antibody proteins of these cells. The relative rates of this incorporation into the proteins of the early and late plasmocytes during the earliest times is low. Hence, the synthesis of proteins and presumably antibody protein is minimal in the mature plasma cells. This gives some support to the suggestion by Fagraeus[1] and Wissler *et al.*[15] that it is the larger immature plasma cells that are producing antibody.

H^3-cytidine

The temporal pattern of labeling in plasmocytic cells after injection of H^3-cytidine was similar to that found after injection of H^3-thymidine, except that the peak increase in the percentage labeling of mature plasma cells did not occur until about 18 hr and was not pronounced. No labeling of the early and late plasmocyte occurred at 0.5 or 1 hr. The pattern and degree of labeling observed is the result of incorporation into both RNA and DNA, since cytidine is a common precursor.[19] Because of the difficulties involved in cytologic identification, no attempt was made to remove selectively either RNA or DNA from the cells before preparation of the autoradiographs. Since there was no evidence of the endogenous synthesis of DNA in the mature plasma cells, and no label was detected in these cells 1 hr after injection of H^3-cytidine, it is concluded that the synthesis of RNA does not occur in the early and late plasmocyte. Consistent with the hypothesized role of RNA in protein synthesis is the finding that only minimal protein synthesis occurs in these same cells. In spite of the fact that the degree and pattern of labeling after the injection of H^3-cytidine is an expression of the synthesis of RNA and DNA, it is interesting that in some cells the sequence of labeling in the nucleus and cytoplasm was what might be expected from the results of *in vitro* experiments.[20-22] One-half hour after injection of H^3-cytidine, the grains were distributed randomly over the nucleus of the labeled early plasmoblasts. By 1 hr, the grains over some of the labeled cells were arranged in small groups over the nucleoli. This arrangement was not found at later times. Exclusively cytoplasmic labeling was observed in some cells 5 hr after injection of the label. This sequence was seen in only a small percentage of the total labeled population and was probably evident only in those cells that were primarily synthesizing RNA during the time the H^3-cytidine was available after injection. Further experiments are in progress in which an *in vitro* system is being used to study the synthesis of RNA and protein by cells of the plasmocytic series.

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QUANTITATIVE ASPECTS OF MATING BEHAVIOR IN DROSOPHILA*

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Introduction

Courtship and mating behavior patterns may be of great importance in establishing sexual isolation between groups of genetically different animals, and thus may have a strong evolutionary influence within a species. Drosophila females, once inseminated, usually do not copulate again for some time. Thus, as far as propagation of that species is concerned, successful copulation with a male from the same species is advantageous. Copulation with a male from another species, however, is of little value if fertile offspring do not result, and might be detrimental in that the female's reproductive capacity would be reduced. Mechanisms that would permit individuals to distinguish between members of their own and alien species would then be of importance in an evolutionary sense. Similarly, differences in mating behavior caused by diverse genotypes within a species might either enhance or decrease the chances for survival of individuals having a particular genotype.

There have been several reports concerning courtship behavior in Drosophila, notably those by Sturtevant,[1,2] Wallace and Dobzhansky,[3] Spieth,[4] Bastock and Manning,[5] Bastock,[6] and Manning.[7] Sturtevant and also Wheeler[8] observed durations of copulations by Drosophila melanogaster, and the number of copulations which male Drosophila could accomplish under varying conditions was reported by Demerec and Kaufmann[9] and Mossige.[10]

It has long been known that preferences exist according to which individuals of one species more often mate with those of the same species than with even closely related ones.[11]

In Drosophila melanogaster Tebb and Thoday[12] observed that individual females had definite preferences as to the mating partner when allowed to choose between two different types of males. Bösiger reported that males having cinnabar-colored eyes had a selective advantage over males having vermilion-colored eyes when both types of males were in the presence of females having vermilion-colored eyes.[13] Hoenigsberg and Santibanez found that inbred males had a marked preference for their own types of females, and that certain outbred strains were indiscriminate in their preference or even preferred alien females.[14] Santibanez and Waddington's investigations showed that white-eyed females were less acceptable to any male than were the other mutant females tested.[15] Petit left white-eyed or heterozygous white/Oregon-R females in the presence of white-eyed and wildtype males. Progeny tests of the females indicated that the genotype of the female did not influence the mating frequency, but that the genotype of the male did.[16] Whether or not the white-eyed

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males had a selective advantage over the red-eyed males depended on the relative proportions of the two types of males in the mating chambers. The selective advantage caused by the white allele also was influenced by the rest of the genetic background. Clise determined that the gene for bar-shaped eyes reduced the mating capacity below that of the wildtype allele and also reduced the fertility of the females carrying the bar gene, thus giving the wildtype flies a selective advantage when competing with bar individuals.[17]

Hoenigsberg, Santibanez, and Sironi investigated sexual preferences between D. prosaltans Duda and D. equinoxiales Dobzhansky and observed that the mating frequency of males with females of their own species was much higher than with females of the other species.[18] Ehrman found that hybrids between two subspecies of D. paulistorum had a genetic background that caused the females to repel the courtship of all males tested, mating with none.[19] The hybrid males courted and were rejected by nearly all the females tested, including their own hybrid siblings.

The experiments reported in this paper describe the mating preferences of D. melanogaster when individual wildtype males were allowed to choose between two females of the same genotype or two females of different genotypes.

Materials and Methods

Two different strains of Drosophila melanogaster were used in these experiments. Wildtype red-eyed males and females were obtained from a Samarkand stock, and the second stock provided the white-eyed females. Both strains had been kept in our laboratory in mass cultures, and no attempt was made to isogenize them.

Cultures of these stocks were emptied of all flies early in the morning, and approximately 3 to 4 hours later, the newly-emerged offspring were collected. During this short period, after emergence of the adults, no copulations would have occurred. The etherized males were immediately placed and stored individually in 4-dr shell vials (19 mm in diameter and 65 mm deep) that contained hardened culture medium about 1 cm deep. The females were stored in similar containers in pairs--two white-eyed or two red-eyed females or one of each type. Early in the afternoon 2 days later, individual males were shaken, without etherization, into the vials that contained the pairs of females. In each experiment approximately 100 of these vials were placed on special observation trays, 45 cm long, 30 cm wide, and 2.2 cm deep. White paper was placed in the tray to allow better observation, and the vials were separated from one another by clear plastic rods and tubes in order to prevent the flies in any vial from being influenced by the activities of flies in adjacent vials. The maximum for each tray was five rows, each containing ten vials.

The time at which each male was placed with its pair of females was recorded, and after the first flies were placed together, observations of the vials were begun at the end of the next 5-minute interval. The flies were then observed in numerical order--always starting with the first in the series--after 5-min intervals throughout the experiment. Since a successful mating usually lasts for an average of 11 or more min, these intervals

were short enough for us to observe the successful copulations, but probably some attempted copulations were missed. Records were made of the premating and intermating periods and the durations of the copulations. When a male had copulated with both females, the vial containing them was removed from the tray. If a male had not copulated with both females within 2 hr after being placed with them, observation of that group was discontinued.

The room temperature varied from 23.4 to 25.8°C during observations, but within individual experiments the range did not vary more than 0.9°C. At all other times, the flies were kept in incubators set at 25°C.

Results

Mating Preference

First Partner

When each of 294 males was given the choice between two red-eyed females as the first mating partner, 90% (264) of the males copulated at least once. When each of 282 males was permitted to choose between two white-eyed females, 95% (264) of the males copulated at least once. When the individual males were given the choice between a white-eyed female and a red-eyed female, 98% (290/297) of the males copulated at least once.

When the males were permitted to choose between the two different types of females, the selection of their first mating partners was not a random choice. The first copulations occurred with white-eyed females in 82% (238/290) of the cases as opposed to only 18% with the red-eyed females.

Second Partner

After the males had copulated for the first time, the mating frequencies with the second females of the pairs were influenced very little by the type of the second female.

Among the 238 males that first copulated with white-eyed females, 81% (192) then copulated with red-eyed females. Of 52 males that copulated first with red-eyed females, 85% (44) then copulated with white-eyed females. When both females in the pair had white eyes, 83% (221) of 268 males that copulated once then copulated with the second female, and when both females of the pair had red eyes 85% (223/264) of the males that copulated once then copulated a second time.

As an indication of the level of activity, a "mating index" may be used. It is defined as the frequency of actual copulations among the possible total of copulations. In the series in which individual males were observed with pairs of red-eyed females, there were 294 males, and if each had copulated with both females, the index would have been 2.00, which is the theoretical maximum for any of the series. However, in this actual series there were 487 copulations, yielding an index of 1.65. The mating index in the white-white series showed an increase to 1.73, and in the series containing a red-eyed and a white-eyed female in each pair, the mating index increased to 1.77. Table I shows the frequencies for

males that did not copulate, copulated once, or copulated twice. It can be seen that even though the white-white and white-red series are similar, the red-red series diverges quite sharply from both.

Table I. Frequency of copulations.[a] Two females and one male present in each vial.

Females present	Neither female copulated (%)	Only one female copulated (%)	Both females copulated (%)	Total males available (number)
Two red	10	14	76	294
Two white	5	17	78	282
One white and one red	2	18	80	297

[a] $\chi^2 = 17.58$, D. F. = 4, $P < 0.01$.

Premating Periods

The interval between the time when the two females and the male were placed together and the first time the male was observed copulating with one of them is taken as the premating period. For comparisons, the copulations were grouped into those that began within the average estimated times of 5, 10, 15, 20, and more than 20 min after the flies were placed together (Fig. 1). The male was not observed when he mounted in each case; therefore, the period is a maximum estimate based on the time the male was first observed copulating.

The males copulated much sooner with the white-eyed than with the red-eyed females. More than 54% of the double copulations with white-eyed females first (WR = 54%; WW = 63%) were initiated within 5 min, but less than 25% of the double copulations with red-eyed females first (RR = 20%; RW = 25%) were begun within this same period.

Intermating Periods

The interval between the times when a male dismounted at the termination of the first copulation and mounted the other female for the beginning of the second copulation is regarded as the intermating period. The exact times when the male dismounted and mounted were not observed. Therefore, an average estimate was calculated from the times when the male was last observed to be copulating with the first female and first observed to be copulating with the second female. The frequencies of individuals within the given intermating periods are presented in Fig. 2. In general, after the first copulation, the males tended to copulate sooner with the second female of the pair if she was a white-eyed rather than a red-eyed female.

Durations of Copulations

The period between the times when a male successfully mounted and dismounted a female is considered as the duration of the copulation. As the male was not observed when he mounted and dismounted in each instance, an average estimate of duration was based on the first and the last times when a male was observed copulating with a particular female.

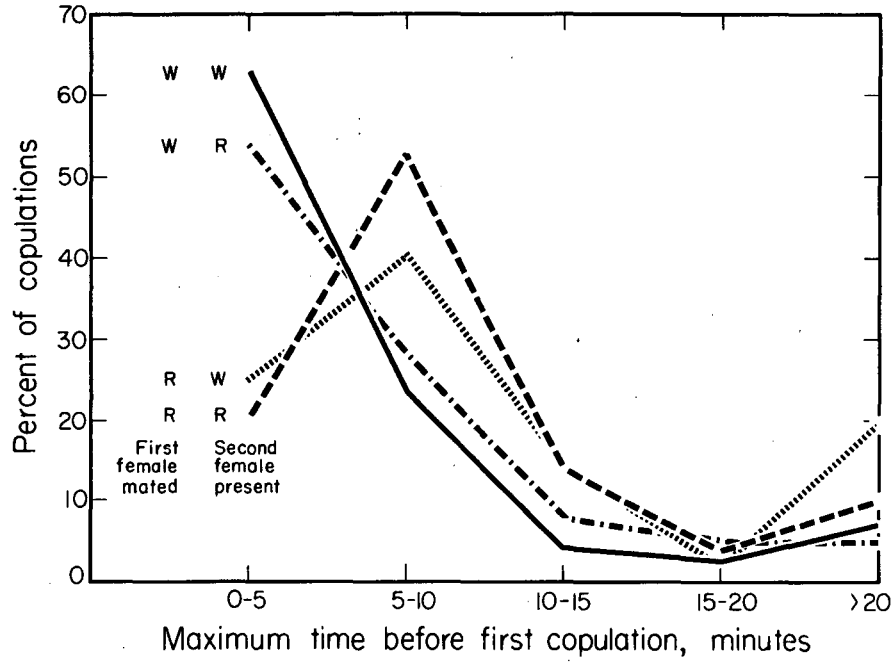

Fig. 1. Premating periods showing per cent of copulations begun within indicated intervals (maximum periods). Number of copulations are WW = 268, WR = 238, RR = 264, and RW = 52.

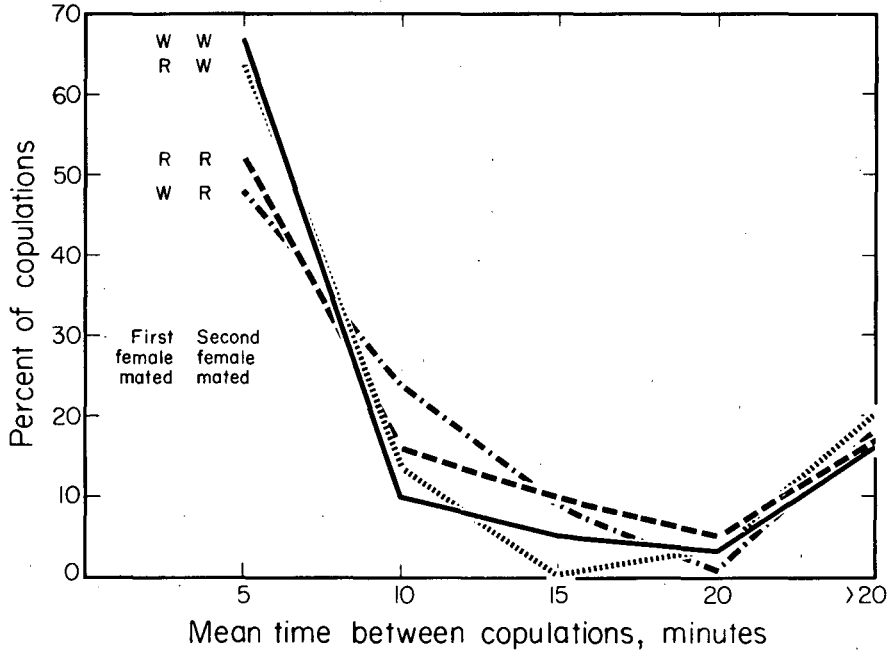

Fig. 2. Intermating periods showing per cent of second copulations by the males begun within indicated intervals (means times used). Number of double copulations are WW = 212, RW = 44, RR = 215, and WR = 193.

Singles Only

The average duration of copulations with the white-eyed females was 13.5 min in the white-white (WW) series and 13.9 min in the white-red (WR) series. The average duration with the red-eyed females was 12.3 min in the red-red (RR) and 11.9 min in the red-white (RW) series. The ranges and distributions of the durations are presented in Table II. The results of the χ^2 analyses of these data are shown in Table III. The average durations with the white-eyed females in each case are greater than either of the averages with the red-eyed females, and although the differences appear to be small, the white-eyed females did copulate for significantly longer periods than the red-eyed (RR vs WR, $P < 0.01$; WW vs RR, $P = 0.02$ to 0.05; WR vs RW, $P = 0.02$ to 0.05). These χ^2 calculations were based on those individuals that copulated for 10 min or less compared with those that copulated for more than 10 min. In one instance (RW vs WW), the differences were not significant, the probability that the differences were caused by chance fluctuations being 0.05 to 0.1. Not much consideration, however, should be given to this or any other calculation involving the RW series because it involved only eight males. There were no significant differences between the series involving white-eyed females ($P = 0.2$ to 0.3) nor those involving red-eyed females ($P = 0.9$ to 0.95) regardless of the type of the second female present.

Table II. Durations of copulations.[a] Per cent of males that copulated for designated intervals.

Females present	Female mated first	Per cent Males copulating (minutes) 5	10	15	20	Total males (number)	Average duration (minutes)
			Singles				
Two white	W	2	28	68	2	47	13.5
Two red	R	0	54	46	0	41	12.3
Red and	W	2	18	80	0	45	13.9
white	R	0	63	37	0	8	11.9
			First of doubles				
Two white	W	1	37	55	7	141	13.5
Two red	R	1	62	35	2	175	11.3
Red and	W	1	39	56	4	193	13.2
white	R	7	45	48	0	44	12.0

[a]Two females and one male present.

First of the Double Copulations

In most cases the averages for the first of the double copulations were not greatly different from the single copulations within their respective mating groups, nor were the ranges and distributions much different from the singles (Tables II and III). The males

copulated for longer times with the white-eyed than with the red-eyed females; in three of the comparisons (WW vs RR, WR vs RW, and RR vs WR), the probabilities that the difference in distributions between the series could be caused by chance fluctuations alone were less than 0.01, and the probability was 0.02 to 0.05 in the fourth instance (WW vs RW).

Table III. Homogeneity tests of differences between singles and first of doubles.

Group	χ^2	Degrees of freedom	Probability
WW	4.34	3	0.2 - 0.3
RR	2.72	3	0.3 - 0.5
WR	10.70	3	0.01 - 0.02
RW	1.20	2	0.5 - 0.7

Second Copulations

The duration of second copulations was generally greater with white-eyed females than with red-eyed females (Tables IV and V). In all cases, the duration of the second copulation was significantly greater than the first of the double copulations ($P < 0.01$). The χ^2 values for white first vs white second are (a) WW vs WW: $\chi^2 = 31.71$, D. F. = 4; (b) WR vs RW: $\chi^2 = 21.51$, D. F. = 3; (c) WW vs RW: $\chi^2 = 11.60$, D. F. = 3. For red first vs red second, the values are (a) RR vs RR: $\chi^2 = 32.68$, D. F. = 4; (b) RW vs WR: $\chi^2 = 15.89$, D. F. = 4; (c) RR vs WR: $\chi^2 = 49.46$, D. F. = 4.

Table IV. Durations of the second copulations.[a] Per cent of males that copulated for designated periods

Females mated		Males copulating (per cent) (minutes)						Total males (number)	Average duration (minutes)
First	Second	5	10	15	20	25	30		
W	W	1	14	58	24	3	0	141	15.6
R	R	1	33	57	8	1	0	175	13.7
W	R	1	28	59	12	0	0	193	14.1
R	W	0	18	59	23	0	0	44	15.2

[a]Two females and one male present.

The durations of the second copulations generally were positively correlated with the durations of the first copulations. A short first copulation would usually be followed by a shorter second copulation than would a longer first copulation (Table VI).

Table V. Homogeneity tests for (A) white second vs red second, (B) white second, and (C) red second.

	Group	χ^2	Degrees of freedom	Probability
A	WW vs RR	24.98	4	< 0.01
	WW vs WR	19.21	5	< 0.01
	RW vs RR	10.82	4	0.02-0.05
	RW vs WR	4.99	4	0.2-0.3
B	WW vs RW	2.16	4	0.7-0.8
C	RR vs WR	4.93	5	0.3-0.5

Table VI. Correlations of the durations of the second copulations with the durations of the first.

Males (number)	Average female copulation time (minutes)		Average male copulation time (minutes)
	White 1st	White 2nd	
1	5	10.0	15.0
53	10	14.7	24.7
77	15	16.0	31.0
10	20	18.0	38.0
	White 1st	Red 2nd	
1	5	15.0	20.0
76	10	13.2	23.3
108	15	14.8	29.8
8	20	16.2	36.2
	Red 1st	Red 2nd	
2	5	10.0	15.0
109	10	12.8	22.8
61	15	15.5	30.5
3	20	13.3	33.3
	Red 1st	White 2nd	
3	5	13.3	18.3
20	10	15.8	25.8
21	15	15.0	30.0

Discussion

Petit concluded that it is the genotype of the male and not of the female that influences the mating frequency.[16] On the other hand, Tebb and Thoday[12] and also Santibanez and Waddington[15] attribute much of the influence on mating frequency to the female. The latter two authors found that white-eyed females were less acceptable to any of the males tested than were any of the other females. Sturtevant had observed that

white-eyed females were chosen as partners more often than were red-eyed females when either red-eyed or white-eyed males were permitted to choose.[1] According to Ehrman, female hybrids between two subspecies of D. paulistorum repelled the courtship of all males tested.[19]

Apparently the mating frequencies may depend on the genotypes of both the males and females. In the experiments reported here, about 82% of the males that copulated at least once did so with white-eyed females first, and only about 18% copulated first with red-eyed females. Observations of individual males with white-eyed and red-eyed females showed that white-eyed females were less active than the red-eyed females, which in most cases would reject the male by running or kicking. The white-eyed females showed less of this avoidance reaction toward the males and with only brief courtship would permit copulation. Frequently males would switch their courtship from the red to the white-eyed females and within seconds would have mounted the white-eyed females for the first copulation.

One small test involved 35 individual white-eyed males, each of which was permitted to choose between a red-eyed and a white-eyed female. The results were similar to those obtained when the red-eyed males were given the same choice. Among the males that copulated, about 80% did so first with the white-eyed females.

The fact that the mating index was greater in the WW and WR series than in the RR series also indicates that the genotype of the female is an important selective factor. The mating index does not distinguish between the single and the double copulations, and therefore statistical analyses were based on the number of males that did not copulate, copulated only once, or copulated twice (Table I). The differences between the WW and the WR series are not great ($P = 0.2$ to 0.3), but the differences between the WR and the RR series are significant at the 5% level. The WR series has an activity level represented by a mating index of 1.77, which is much greater than that of the RR series, in which the mating index was 1.65 ($P < 0.01$, $\chi^2 = 16.42$, D. F. = 2).

After the males had copulated once, the types of the second females in the vials had little influence on whether or not the males would copulate a second time. Approximately 81 to 85% of the males copulated a second time regardless of the types of females with which they had copulated first, and regardless of the types of the other females in the vials. In each case, analyses of these data yield probabilities of at least 50% that the differences could have been caused by chance fluctuations.

Premating Periods

The fact that the males copulated much sooner with the white-eyed than with the red-eyed females (Fig. 1) is a direct result of the lesser avoidance reaction of the white-eyed than the red-eyed females toward the males. When the males were permitted to choose between two white-eyed females, 63% copulated within 5 min from the time that the flies were placed together. Yet, during the same interval, only 20% of the males given a choice between two red-eyed females copulated. The probability that these differences

would have occurred by chance alone is less than 0.01 (χ^2 = 102.73, D. F. = 1). When the individual males were permitted to choose between a white-eyed and a red-eyed female, 54% copulated with white-eyed and only 25% with red-eyed females during this 5-min period. As in the previous analysis, the probability that chance alone would have accounted for the differences is less than 0.01 (χ^2 = 13.96, D. F. = 1). The two remaining analyses of the difference between the mating frequencies within the first 5 min also show that the males copulated sooner with the white-eyed than with the red-eyed females, and the differences are very significant (WW vs RW: χ^2 = 25.47, D. F. = 1; RR vs WR: χ^2 = 63.34, D. F. = 1).

When the males chose red-eyed females as their first partners, the type of the second female present in the vial had little influence on the frequency of copulations within the first 5 min. Twenty per cent of the males in the RR series and 25% in the RW series copulated with red-eyed females within this period (P = 0.3 to 0.5, χ^2 = 0.7449, D. F. = 1). Apparently the males courted the red-eyed females only, or perhaps in these cases the white-eyed and red-eyed females had similar avoidance reactions.

When the males chose white-eyed females as their first partners, the type of the second female present did influence the frequency of copulations within the first 5 minutes. During this period, there were significantly more copulations within the WW than in the WR series (P = 0.02 to 0.05, χ^2 = 4.40, D. F. = 1). This may have been because the males courted unsuccessfully the red-eyed females before copulating with the white-eyed ones.

Intermating Periods

The average intermating periods before the copulations were shorter with the white-eyed females in each instance than with the red-eyed females (Fig. 2). The differences in distributions were found to be highly significant when the WW was compared with the RR group (P < 0.01, χ^2 = 17.76, D. F. = 5) and when the WW was compared with the WR series (P < 0.01, χ^2 = 32.12, D. F. = 5). In the other comparisons, WR vs RW and RR vs RW, the differences were not significant. The RW series, however, contained less than a quarter as many double copulations as any of the other three groups, and the statistics involving this group are not reliable. The type of female with which the male mated first had little influence on the intermating periods, as the differences in distributions between the WW and RW series and between the RR and WR series were not significant.

The shorter intermating periods before the copulations with the white-eyed females than with the red-eyed females are correlated also with the tendency of the red-eyed females to actively repel the courtship of the male.

There is an interesting correlation between the premating and intermating periods (Fig. 3). In the WW series, 67% of the first of the double copulations were begun within 5 min after the flies were placed together. Within 5 min after the termination of the first copulations, 67% of these males had begun a second copulation (Fig. 3a). The premating periods before each of the two successive copulations follow similar distribution patterns

(Fig. 3a), and, based on the frequencies of copulations initiated within the first 5 min in each of these categories, the differences are not significant (P = 0.99+). The differences between pre-WR (56%) and inter-RW (62%) are hardly significant, as seen in Fig. 3b, nor are the differences between pre-WW (67%) and inter-RW (62%). The probabilities for chance deviations accounting for the differences in the two latter cases are respectively 0.3 to 0.5 and 0.5 to 0.7.

Apparently, whether or not a male had copulated previously had little influence in determining the durations of the periods before either the first or second copulations with white-eyed females. The periods before the copulations with the red-eyed females, however, were greatly influenced by whether or not a copulation had occurred previously.

Distribution patterns for the periods before the first copulations with red-eyed females differed from the patterns for the periods before the second copulations with red-eyed females (Fig. 3c, d). When the males had not copulated previously, only about 20% (pre-RR, Fig. 1c) to 27% (pre-RW, Fig. 3d) of the first copulations with the red-eyed females were begun within the first 5 min after the flies were placed together. However,

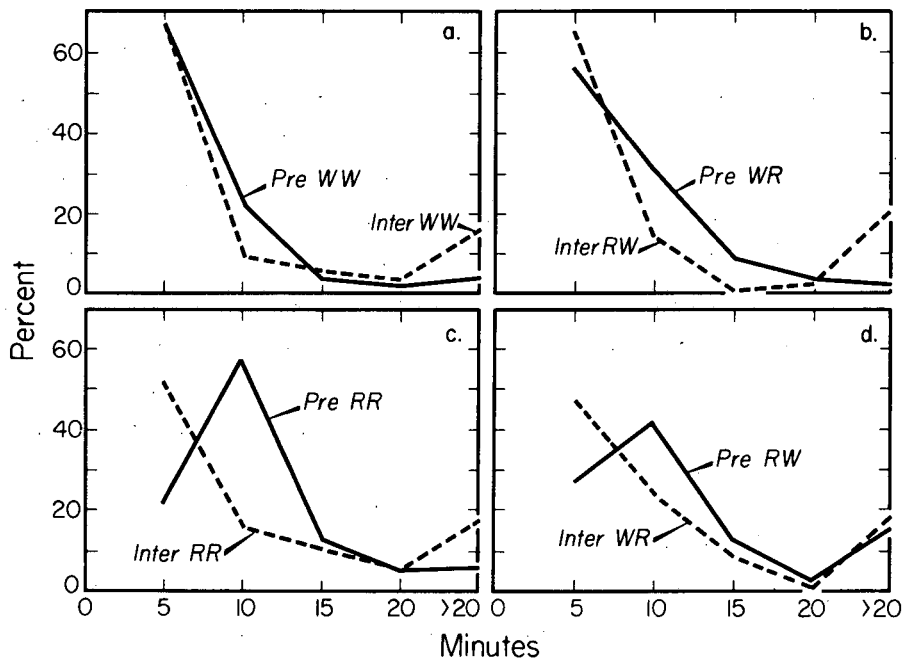

Fig. 3. Comparison of premating and intermating periods before copulation with white-eyed females (a and b) and before copulation with red-eyed females (c and d). Per cent of males copulating within specified intervals are shown. Numbers of double copulations are WW = 212, RW = 44, RR = 215, and WR = 193. Maximum times used for premating and meantimes for intermating periods are given.

if the males had each copulated once, then about 48% (inter-WR, Fig. 3d) to 52% (inter-RR, Fig. 3c) of the second copulations of the males were begun within 5 min after the

termination of the first. In each case the distributions, based on the frequencies of copulations within the first 5-min intervals of the premating as opposed to the intermating times, are significantly different from each other ($P < 0.01$, D. F. = 1; $\chi^2 = 46.88$, for pre-RR vs inter-RR, $\chi^2 = 6.93$ for pre-RW vs inter-WR). A third test, comparing the premating periods of the RR series with the intermating periods of the WR series, also yielded results that are highly significant ($\chi^2 = 35.29$, D. F. = 1).

One possible explanation for why the males mated sooner with the red-eyed females after previous copulations is that a chemical released during the copulation in some way increased the mating drive of either the red-eyed female or the male. The former is more likely, because the premating and intermating times before copulations with white-eyed females hardly differed from each other. The type of female with which the male had copulated previously was not important in influencing the intermating periods, suggesting that the same chemical stimulus is produced in the copulations with each type of female. This would also suggest that the white-eyed females do not react as strongly to the stimulus as do the red-eyed females.

Mayr showed that a chemical that affects the mating threshold of the female is released by the male.[20] Antennae (containing olfactory organs) were removed from female Drosophila. Males of one or more types were placed with these females, and observations of their mating behavior were made or, in some instances, fertility of the females was used as the criterion to indicate mating activity. Petit, on the other hand, minimized the role of chemical receptors of the females.[21] Her experiments involved excision of antennae and aristae from female D. melanogaster and then observing courtship behavior of these and nonoperated females with red-eyed and white-eyed males. The perception, by organs within the antennae and aristae, of differences in the patterns of the wing vibrations of the two types of males accounted for the higher frequency of red-eyed than white-eyed males being chosen as partners. Sturtevant observed that a pair of flies would mate sooner if placed in a vial in which a copulation had just occurred than would a pair placed in a clean vial.[1] This would indicate that olfaction and not perception of differences in wing-vibration patterns was the important factor.

The work presented here indicates that olfaction is important, at least in determining the frequency of second copulations within the same mating vials, but the perception of differences in wing-vibration patterns is not entirely ruled out.

Duration of Copulations

The white-eyed females generally copulated for longer periods than did the red-eyed females regardless of whether the copulation was the first, second, or only one accomplished by the male. The white-eyed females may be capable of copulating for longer times than the red-eyed females, and it is also possible that the duration is determined by the male, either directly or indirectly. The red-eyed females might force the males to dismount, or the males might react to the copulations with the red-eyed females by dismounting earlier, even though the duration could be prolonged. Another possibility

is that insemination of the red-eyed females may deplete the sperm supply faster than does the insemination of the white-eyed females, thus correlating the duration of the copulation with the sperm supply.

The duration of copulation seems to depend on the male to a large extent. Some copulate for short periods, while others copulate for greater ones. This role of the male is indicated by the fact that the average duration of the second copulation was greater than the first, regardless of the duration of the first. Scatter diagrams indicating correlations of the duration of the second with the first copulation are presented in Figs. 4 and 5. The correlations are positive in each case, though extremely slight in the RW series (Fig. 4d). In the other series, the correlation coefficients do not differ much from each other. Even though the correlation coefficients are not large, in each of the series except the RW, the

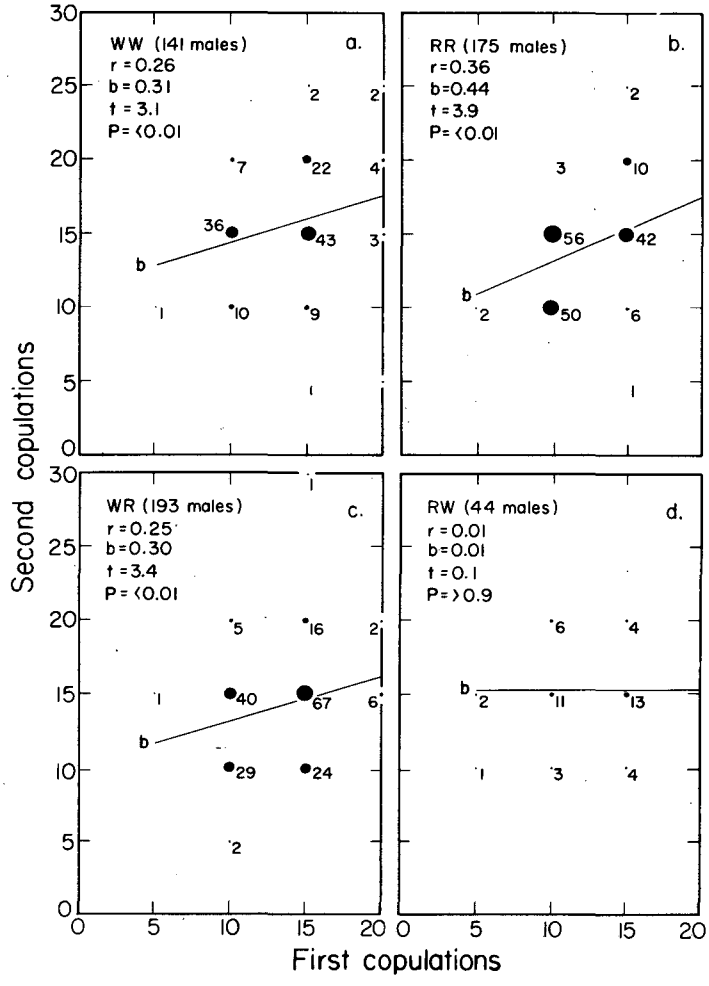

Fig. 4. Duration in minutes of first and second copulations. Here r is the correlation coefficient, and b is the regression of second copulations dependent on first. Area of the dots is approximately proportional to the numbers of individuals.

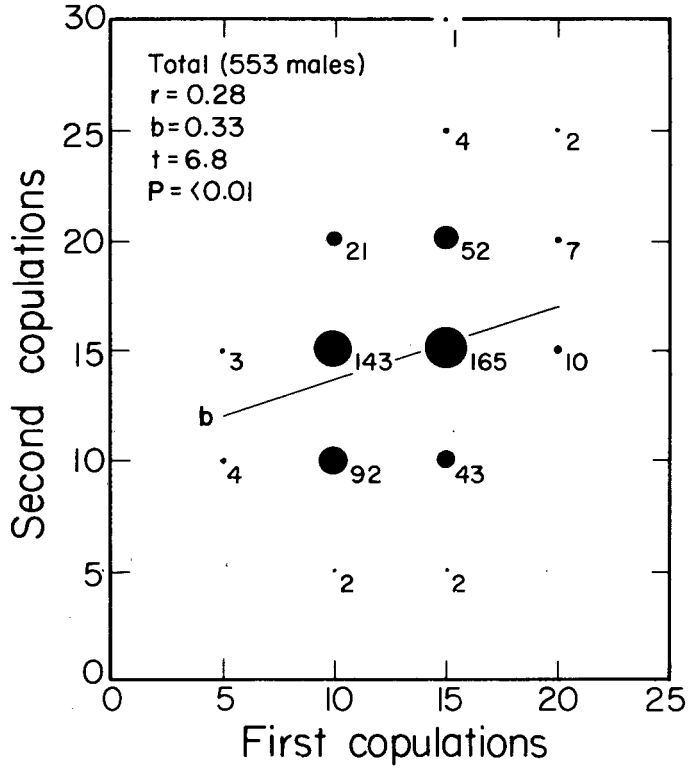

Fig. 5. Duration in minutes of first and second copulations. Total data 4 a-d. Here r is the correlation coefficient, and b is the regression of second copulations dependent on first. Area of the dots is approximately proportional to the numbers of individuals.

positive increases in the duration of the second copulation dependent upon the increases in the duration of the first were significant (P = 0.01). The low correlation in the RW series may be a result of the small number of individuals in the sample.

Acknowledgments

The author wishes to thank Miss Cole Brunt for technical assistance and for carrying out the statistical analyses. The author also wishes to thank Dr. Curt Stern for advice throughout the course of the experiment, and Dr. Stern and Dr. Peter Marler for their valuable suggestions during preparation of the manuscript.

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RADIATION-CHEMICAL REACTION IN BIOCHEMICAL SYSTEMS

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Winifred Bennett, and Sibyl A. Cole

Studies of the indirect action of ionizing radiation on protein have rather generally emphasized the reactions of chemical bonds and groups that can be measured through direct application of the analytical methods of conventional protein chemistry. Although this approach has provided information on the "radiation sensitivity" of various of the more labile bonds, particularly those associated with the sulfur moieties and the aromatic and heterocyclic configurations, it has per se but limited application as a general approach to the radiochemical study of protein systems. Work carried on at this Laboratory over the past several years has established (a) that chemical groups not ordinarily associated with protein systems are introduced by both the direct and indirect actions of ionizing radiation, and (b) that formation of such groups can account for a large fraction of the total absorbed energy.[1-4] These studies have necessarily involved the development of specialized analytical procedures that have been used adjunctively with other methods.

The evidence we have obtained to date generally supports the concept (a) that major chemical effects of both the direct and indirect action of ionizing radiation on protein involve reactions at C-N bond loci, (b) that these reactions under appropriate conditions can lead to C-N bond cleavage, and (c) that the fundamental characteristics of the cleavage reactions are essentially independent of the nature of the group or linkage with which the C-N bond is associated. These considerations are summarized by the generalized overall reactions given below.

Oxygenated solution:

$$R'NHCH(R'')R''' + O_2 + H_2O \longrightarrow R'NH_2 + R''COR''' + H_2O_2 \qquad (1)$$

Evacuated solution:

$$R'NHCH(R'')R''' + H_2O \longrightarrow \left[(R'N{=}C(R'')R''' + H_2O)\right] + H_2 \qquad (2)$$

$$\downarrow$$

$$R'NH_2 + R''COR'''$$

Evacuated solid:

$$R'NHCH(R'')R''' \longrightarrow R'N{=}C(R'')R''' + H_2 \qquad (3)$$

$$[+ \downarrow H_2O]$$

$$R'NH_2 + R''COR'''$$

It is noted that a carbonyl-containing product is common to all these cleavage reactions. The nature of the corresponding nitrogen product, on the other hand, is determined by the configuration of the parent nitrogen-containing function. Amino groups (R' =H), for example, yield ammonia, while secondary amine linkages are degraded to primary amine, and so forth. The acylamine linkage (R' =RCO) is naturally of

fundamental interest in that it characterizes the peptide bond and represents the single most recurrent configuration in the protein molecular structure.

Oxygenated Solution

Rewriting Eq. (1) for a typical peptide model, such as an acylamino acid, we have

$$\mathrm{RCONHCH(R)COOH} + O_2 + H_2O \longrightarrow \mathrm{RCONH_2} + \mathrm{RCOCOOH} + H_2O_2. \qquad (1a)$$

The principal intermediate processes in oxygenated solution involve the initial radiation-chemical step[5]

$$H_2O \rightsquigarrow H,\ OH,\ H_2,\ H_2O_2, \qquad (4)$$

followed by

$$H + O_2 \longrightarrow HO_2, \qquad (5)$$

$$OH + \mathrm{RCONHCH(R)COOH} \longrightarrow H_2O + \mathrm{RCONHC(R)COOH}, \qquad (6)$$

$$H_2O + O_2 + \mathrm{RCONHC(R)COOH} \longrightarrow \mathrm{RCONH_2} + \mathrm{RCOCOOH} + HO_2, \qquad (7)$$

$$2\ HO_2 \longrightarrow H_2O_2 + O_2. \qquad (8)$$

Reaction (7) may possibly involve a short-lived organic hydroperoxide intermediate, but the point of interest here is that the amide and carbonyl functions appear as initial reaction products, and this fact is most simply represented by the indicated reaction. We have studied this radiation-induced reaction of the peptide linkage in oxygenated solutions of acylamino acids, aminoacid anhydrides, simple peptides, and protein. In irradiated protein systems, both the amide and carbonyl functions should of course appear, on the basis of Reaction (1a), as terminal groups of high-molecular-weight protein fragments,

$$\sim\sim\mathrm{CONHCH(R)}\sim\sim + O_2 + H_2O \longrightarrow \sim\sim\mathrm{CONH_2} + \mathrm{RCO}\sim\sim + H_2O_2. \qquad (1b)$$

That high-molecular-weight carbonyl compounds are formed as initial irradiation products in oxygenated protein solutions has been demonstrated by use of the carbonyl reagent 2, 4-dinitrophenylhydrazine (2, 4-DNPH). The irradiated protein solutions are treated with 2, 4-DNPH and are then dialyzed to separate excess reagent and any products of low molecular weight. Control runs with unirradiated solutions have shown for most of the proteins studied only a negligible retention of 2, 4-DNPH reagent. In any case, the absorption spectra of the hydrazone products (in methanol–potassium hydroxide solution) are characteristic of the $>C{=}N\text{-}NH\text{-}C_6H_3(NO_2)_2$ chromophore and are readily distinguished from the reagent spectrum. Quantitative applications of this colorimetric procedure[6] give (initial) G values of about 1.0 to 1.2 for total carbonyl production in gamma-irradiated solutions of pepsin and gelatin. A modified procedure[7] in which excess reagent is extracted with ether and then assayed to give G(>CO) in terms of 2, 4-DNPH consumption gives essentially identical results. Although most of our studies have employed protein of low sulfhydryl content, measurable reaction has also been observed with typical "sulfhydryl" proteins. For example, under identical conditions the initial G(>CO) value for alcohol dehydrogenase is about 0.5.

To obtain detailed information about the positions of these carbonyl groups, it would be helpful if the protein hydrazones could be hydrolyzed directly to elementary compounds that could then be identified separately. But, unlike the classical case of the dinitrophenylamino acids,[8] we find, as might be expected, that the dinitrophenylhydrazones undergo extensive decomposition and rearrangement under the experimental conditions employed generally for protein hydrolysis. On the other hand, control runs have established that the free carbonyl compounds themselves are stable under the conditions of hydrolysis employed (2 N HCl at 90°C for 18 to 24 hr). Transamination reactions, for example, do not appear to introduce any serious analytical problems.[9] Hence, in our identification studies, the irradiated solutions have been acid-hydrolyzed first, then passed through a column of Dowex-50 resin (hydrogen form), and finally treated with 2, 4-dinitrophenylhydrazine reagent. Since Dowex-50 removes nitrogen-containing hydrolysis products, including any nitrogen derivatives of α-keto acids, the effluent solution contains only α-keto acids derived from neutral and acidic amino acids. Methods for the separation and determination of nitrogen-substituted α-keto acids derived from basic amino acids (lysine, etc.) are being developed.

After treatment with reagent, the hydrazone compounds are extracted with chloroform and examined chromatographically. With gelatin, for example, we find α-keto glutaric, glyoxylic, pyruvic, and phenyl pyruvic acids as major components of this α-keto acid fraction. Various difficulties have been experienced in attempting to measure yields of the α-keto acids with acceptable precision. Each α-keto acid hydrozone can exist in two isomeric forms, and these not only are formed in variable relative yield (as influenced by temperature, solvent, etc.) but also have different R_f values and spectrophotometric properties.[10] Since isomerism in these compounds is attributed to hydrogen bonding between N-H of the imino group and C=O of the carboxyl group, we investigated the use of α(2, 4-dinitrophenyl) α-methyl hydrazine[11] as a reagent for α-keto acids. No evidence for isomerism in the hydrazones prepared with this hydrazine derivative has been observed. Incidentally, it should also be noted that control analysis of an unirradiated protein may show traces of pyruvic and α-keto butyric acids. These arise respectively from decomposition of serine and threonine during the hydrolysis step.[9] Most of our α-keto acid studies have been made with gelatin that has a relatively low serine-threonine content, and show a negligible α-keto acid background.

Measurements of ammonia and amide production in oxygenated protein solutions are also generally consistent with the mechanisms outlined. Here again we find gelatin (lime-processed) to be particularly suitable for study because of the low amide content of this derived protein. Analytical methods employed in the measurement of amide yields are based on standard hydrolytic procedures.[12] The rate of ammonia liberation from irradiated gelatin solutions (as with irradiated solutions of simple peptides) appears to be characteristic of the amide group. Hydrogen peroxide and organic peroxides are apparently not involved in the postirradiation reaction. Initial G values for formation of free ammonia and "amide-like" ammonia are respectively about 0.3 and 1.3. It is likely that

the free ammonia comes predominantly from terminal and (or) side-chain amino groups. Deamination of the lysine residue, for example, would yield ammonia and an aldehydo α-amino-acid,

$$\overset{\wr}{\underset{\wr}{N}}-(CH_2)_n-CH_2NH_2 + O_2 + H_2O \longrightarrow \overset{\wr}{\underset{\wr}{N}}-(CH_2)_n-CHO + NH_3\cdot + H_2O_2.$$

Corresponding reaction at the arginine residue would liberate guanidine,

$$\overset{\wr}{\underset{\wr}{N}}-(CH_2)_n-CH_2-NH-\underset{\underset{NH}{\|}}{C}-NH_2 + O_2 + H_2O \longrightarrow \overset{\wr}{\underset{\wr}{N}}-(CH_2)_n-CHO + NH_2-\underset{\underset{NH}{\|}}{C}-NH_2 + H_2O_2,$$

which, under the analytical conditions employed, would contribute to the yield of "amide" ammonia. Methods for the separation and determination of possible aldehydo α-amino-acids and amino α-keto acids (referred to earlier) are being developed.

Oxygen-Free Solutions

If, after irradiation, an oxygen-free protein solution is treated with 2, 4-DNPH and then dialyzed, negligible retention of the reagent is observed. This result is, of course, quite different from that obtained if dissolved oxygen is present during radiolysis. However, if the irradiated solution is hydrolyzed first and then analyzed, we find that the α-keto acid (and ammonia) yields approach those observed in the corresponding treatment of the oxygenated systems. Similar results have been obtained with a number of simple peptides and have been interpreted in terms of the formation of imino (dehydro) derivatives as defined by the over-all Eq. (2). For reactions at the peptide bond, this equation takes the form

$$RCONHCH(R)COOH \longrightarrow RCON{=}C(R)COOH + H_2, \qquad (2a)$$

and the postirradiation hydrolysis corresponds to

$$H_2O + RCON{=}C(R)COOH \longrightarrow RCONH_2 + RCOCOOH. \qquad (2b)$$

The intermediate processes are formulated as

$$H + RCONHCH(R)COOH \longrightarrow RCONH\dot{C}(R)COOH + H_2, \qquad (9)$$

$$OH + RCONHCH(R)COOH \longrightarrow RCONH\dot{C}(R)COOH + H_2O, \qquad (6)$$

followed by the radical disproportionation reaction to give the dehydropeptide product,

$$2\ RCONH\dot{C}(R)COOH \longrightarrow RCONHCH(R)COOH + RCON{=}C(R)COOH. \qquad (10)$$

Also included here for purposes of subsequent discussion is the alternative radical combination reaction,

$$2\ RCONH\dot{C}(R)COOH \longrightarrow \begin{array}{l} RCONHC(R)COOH \\ \quad\ \ | \\ RCONHC(R)COOH, \end{array} \qquad (10a)$$

because, as will be shown, products in addition to those leading to cleavage of the C-N bond are involved.[13]

Now, the dehydropeptide configuration has been well characterized because of its relationship to the chemistry of the unsaturated azlactones.[14] Numerous dehydro derivatives, such as the acetyldehydroamino acids, etc., have been prepared, and generally speaking, these are quite stable in aqueous solution under ordinary conditions. Boiling in dilute mineral acid, however, readily brings about hydrolysis. By and large, the theory that dehydropeptides are formed by the indirect action of radiation on peptides in oxygen-free solution is wholly consistent with the chemical properties of the known dehydropeptides.

Further, the stability of the dehydro compounds would suggest that direct chemical evidence of their formation, in solutions of the simpler peptides at least, might be obtained. Accordingly, we initiated a detailed radiochemical study of oxygen-free acetylglycine solutions to determine, among other things, the relative contributions of the disproportionation and combination reactions that in this system would give acetyldehydroglycine and acetyl diaminosuccinic acid (i.e., Reactions (10), (10a) respectively).

The acetylglycine was prepared by heating stoichiometric amounts of acetic anhydride and C^{14}-labeled glycine in glacial acetic acid.[15] Even under optimum conditions, some cyclization of acetylglycine to the azlactone occurs. Purification and separation of the $CH_3CONHCH_2C^{14}OOH$ was effected by partition chromatography on a silicic acid column. Elution was carried out with chloroform-butanol mixtures; the details of the method have been given.[13, 16] Acetylglycine is separated completely from the azlactone under such conditions. The purified $CH_3CONHCH_2C^{14}OOH$ in 0.1 M solution was irradiated under previously described conditions with Co^{60} gamma rays from a 100-curie source. An aliquot of the irradiated solution was hydrolyzed to elementary compounds in 4 N hydrochloric acid (*in vacuo*) for 24 hr, evaporated to dryness at room temperature under reduced pressure, and then analyzed chromatographically on Dowex-50 (hydrogen form). Hydrochloric acid in progressively increasing concentration (0 to 4 N) was used to elute fractionally the nitrogen products. A typical elution curve is shown in Fig. 1. The C^{14} activity associated with peak I passes through the column with little or no retention. When this fraction was subsequently cochromatographed with added authentic glyoxylic acid on a silicic acid column, an exact correspondence between C^{14} activity and titer was obtained. The major peak III of Fig. 1 corresponds, of course, to $NH_2CH_2C^{14}OOH$ released from the parent compound during hydrolysis. Product fractions II and IV have been identified respectively as aspartic and diaminosuccinic acids. When aliquots of each fraction were rechromatographed with the appropriate authentic material, exact correspondence between activity and titer of the hydrochloride salts was observed. Incidentally, the characteristic double peak of the diaminosuccinic acid fraction (IV) results from the separation of isomeric forms.[13]

Now, if glyoxylic acid (and ammonia) does arise from the acid hydrolysis of acetyldehydroglycine as proposed, it should be possible to isolate the dehydro intermediate from

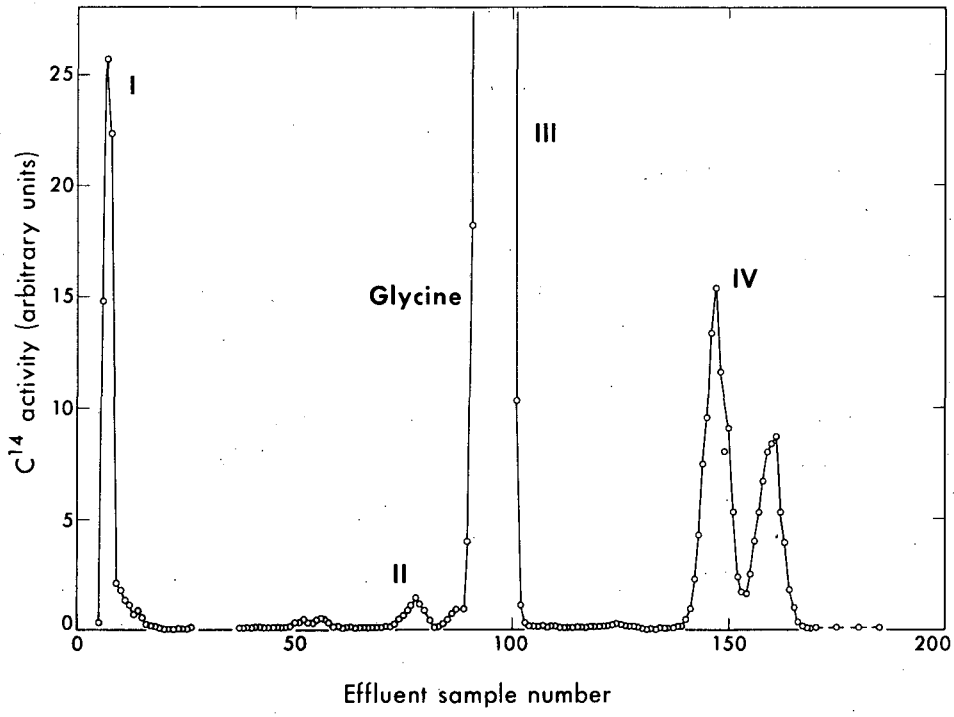


Fig. 1. Elution curve (Dowex-50) for irradiated 0.1 M acetylglycine ($CH_3CONHCH_2C^{14}OOH$) after hydrolysis.

the unhydrolyzed target solution, since, as mentioned earlier, these compounds are known to be generally stable in aqueous solution under ordinary conditions. Of the experimental methods available, it seemed that partition chromatography on silicic acid offered the best possibilities for the direct isolation and characterization of compounds of this class. Accordingly, an irradiated $CH_3CONHCH_2C^{14}OOH$ solution was chromatographed directly on a silicic acid column with our standard chloroform-butanol solvent sequence. Figure 2 shows the elution curve in terms of C^{14} activity. The first point of interest is that the glyoxylic acid region (peak I) shows relatively little activity. Secondly, it is readily seen that the total C^{14} activity associated with the separated peaks I, III, and IV of Fig. 2 represents only about half the C^{14} activity found in the glyoxylic and diaminosuccinic peaks after hydrolysis, as shown in Fig. 1 (the data of Figs. 1 and 2 have been normalized for purposes of direct comparison). The simplest explanation, of course, is that the acetylglycine peak of Fig. 2 also contains acetyldehydroglycine. Hydrolysis studies of the material associated with peak II are now in progress. Peaks III and IV have been found to involve primarily the acetyl derivatives of diaminosuccinic acid.

Although dehydrogenation via Reaction (9) is represented as the major path for H atom removal in oxygen-free solutions of the simpler peptides, we have recently found evidence that some H atom addition to the C=O group of the peptide linkage also occurs. The types of process to be described were first detected in oxygen-free solutions of the aminoacid anhydrides (diketopiperazines). For example, if an oxygen-free solution of

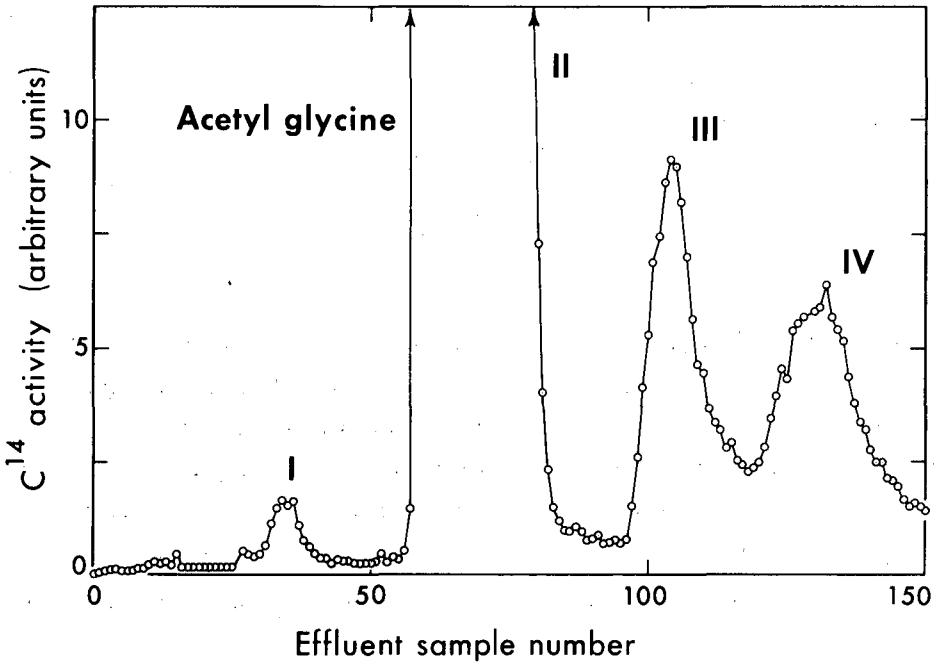
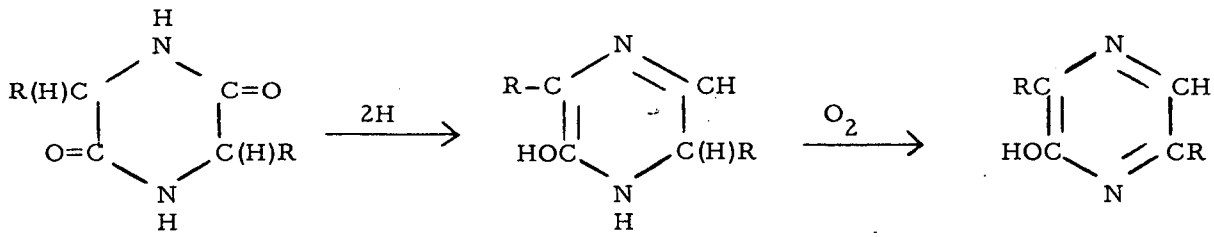


Fig. 2. Elution curve (silicic acid) for irradiated 0.1 M acetylglycine ($CH_3CONHCH_2C^{14}OOH$) prior to hydrolysis.

alanine anhydride is irradiated and examined spectrophotometrically, the differential spectrum (read against unirradiated solution) shows negligible absorption above 260 mμ as long as the irradiated solution is not exposed to the atmosphere. However, if oxygen is introduced, then absorption above 260 mμ gradually increases, and the rate of increase is accelerated by addition of either acid or base. The absorption maximum in acid solution is at about 340 mμ; in basic and neutral solution it corresponds to 320 mμ. The position of the absorption maximum shifts reversibly with change in pH, and the evidence is that the nature of the oxidation product is the same regardless of the pH of the solution during the postirradiation step. Irradiated solutions of glycine anhydride exhibit similar properties except that the wave length of maximum absorption (320 mμ) is independent of pH. It should be noted also that these postirradiation phenomena are not observed if oxygen is present during irradiation.

After some speculation it occurred to us that a rather straightforward interpretation of the postirradiation effects in aqueous diketopiperazine systems could be formulated. In general terms, it involves a radiation-induced reduction of the diketopiperazine molecule (I) to the corresponding 1, 2-dihydropyrazine derivative (II) which is of a class of compounds known to be readily oxidized[17] (e.g., by air) to the corresponding pyrazine structure (III).

Isolation of 2-hydroxy-3, 6-dimethyl pyrazine from an irradiated alanine anhydride solution has recently been accomplished. The solution was "freeze-dried" and taken up in 25 ml of methanol. An appropriate aliquot was analyzed chromatographically on filter



(I) (II) (III)

paper with a butanol-ammonia solvent system. At low dosages of irradiation a single fluorescent component ($R_f \approx 0.7$) was observed. This was eluted and compared spectrophotometrically with authentic[18] material. Figure 3 shows a normalized plot of the data obtained in the two cases. Subsequent cochromatographic studies further support the identification. The yield of the pyrazine derivative from 0.1 M alanine anhydride solution corresponds to a G value of ≈ 0.4 (Co^{60} gamma rays, 7×10^{19} ev/ml).

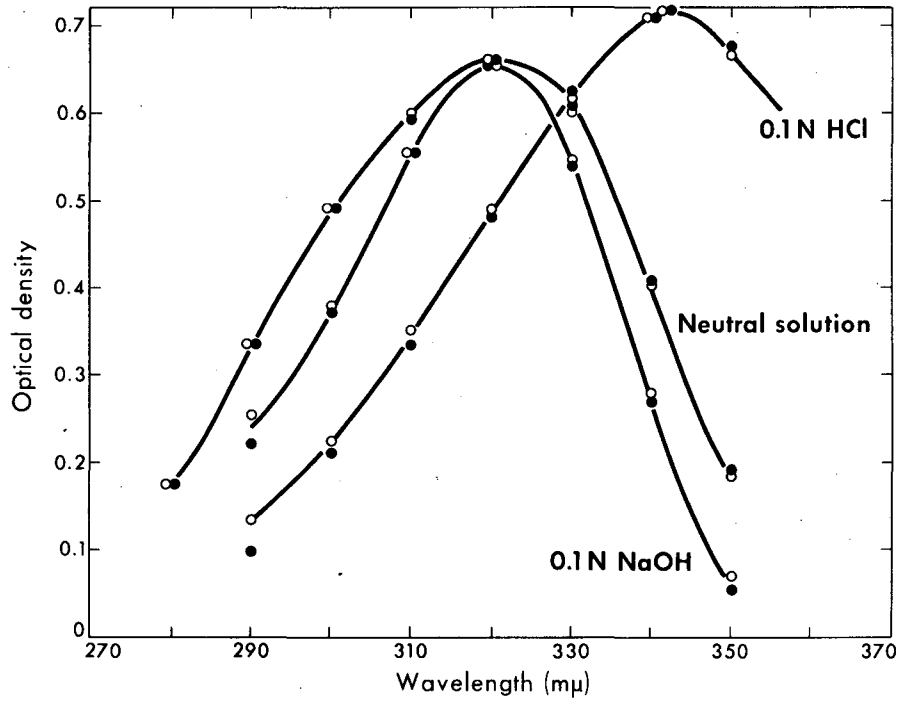


Fig. 3. Absorption curves of authentic 2-hydroxy-3, 6-dimethylpyrazine (O) and of product (●) isolated from irradiated 0.1 M alanine anhydride.

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SOME PHYSICOCHEMICAL ASPECTS OF ERYTHROPOIETICALLY ACTIVE PLASMA*

Howard C. Mel and Milorad M. Radotić

Introduction

In the extensive biological (and recently chemical) studies on erythropoietic "hormone" activity (EPH), in numerous laboratories around the world over the past half century, a variety of contradictory results has been reported. Even today uncertainty or disagreement exists as to the site of origin, target organ, and physicochemical nature of the active molecule, and even as to the existence of one or more active species. Major factors probably contributing to these discrepancies are differences in source materials, in physicochemical treatment, and in assay methods, as summarized in Fig. 1.

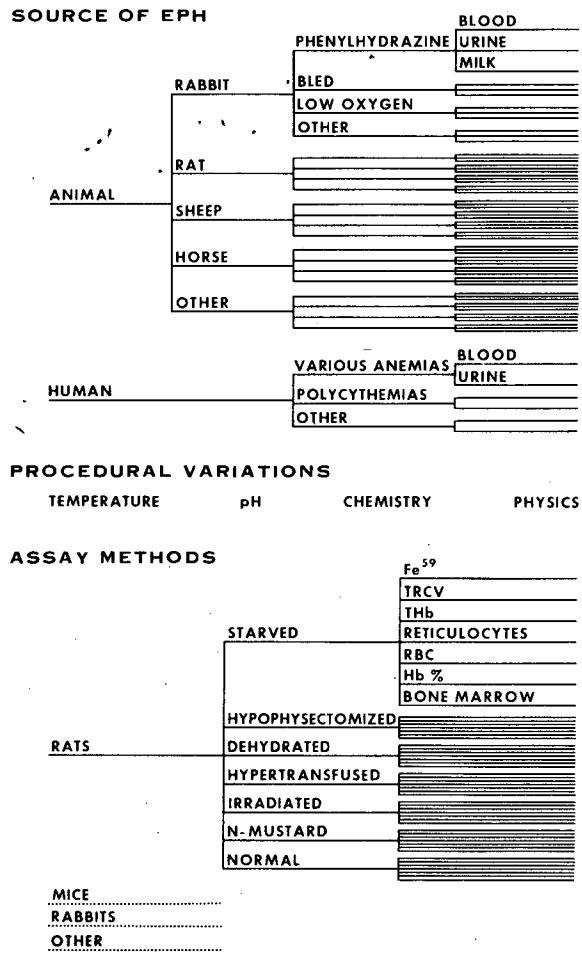

Fig. 1.

*Based on a paper delivered before the American Federation for Clinical Research, Western Section Meeting, Carmel, California, Jan. 26, 1961. See Clin. Res. 9, 66 (1961).

A variety of source materials has been employed, both animal and human. The most common animals are rabbits, sheep, and rats, though others also have been used. The methods for inducing anemia (and EPH activity) include phenylhydrazine treatment, bleeding, and exposure to low oxygen pressure. The most common biological fluids employed are blood and urine, though milk and other fluids also have been used by some workers. For human source material blood and urine have been the principal fluids. The really high human activities have generally been associated with severe anemia, though activity has been noted in polycythemia and other conditions. (See the extensive review by Gordon.[1])

With these different starting materials, a variety of temperature, pH, and general physical and chemical conditions have been employed.

For assay purposes animals (commonly rats) may be normal, but more often have erythropoiesis reduced by starving, hypophysectomy, dehydration, hypertransfusion, irradiation, or chemical treatment. Activity has been measured by determination of Fe^{59} uptake in circulating erythrocytes, total red cell volume, total hemoglobin, reticulocyte count, red blood cell count, and hemoglobin g%, and by bone marrow examinations, among others. A variety of injection schedules is also commonly employed. Not all combinations of these factors have been tried, of course, but there is a rather staggering diversity of common practice.

An important clarification of the assay method was published in October 1960 as a cooperative effort of seven laboratories.[2] These workers all assayed portions of an active rabbit plasma filtrate, CS-1, prepared by the group at California Institute of Technology, providing for the first time a widespread multiple interlaboratory assay comparison. Five of the seven groups used a form of the Fe^{59} assay, and the joint proposal was to define future activity assays in terms of the Fe^{59} uptake in circulating erythrocytes in starved rats, until such time as a standard material might be widely available. We have adopted this convention in the work presented here.

There still remain important differences in starting materials and in subsequent chemical and physical handling procedures, which of course can affect the activity. We will report herein on some of our work on these physicochemical aspects as they relate to the biological activity of plasma from phenylhydrazine-anemic rabbits, and plasma of a patient with secondary polycythemia.

Initial Treatments

To aid in the discussion we have collected in Fig. 2 some representative treatments used by various workers with erythropoietically active plasma. The procedure of Borsook, Graybiel, Keighley, and Lowy, of boiling acidified plasma,[3] is widely used to reduce antigenicity for long-term injection experiments as well as for partial purification purposes, since the activity remains in the supernatant. There is general agreement that long-term boiling certainly leads to major activity loss, but reports on short-term boiling are more ambiguous. There appears to be some decrease in Fe^{59} uptake upon 5 to 10 min boiling,

INITIAL TREATMENT OF ERYTHROPOIETICALLY ACTIVE STARTING MATERIAL

BGKL	Anemic rabbit plasma	HCl : boiling : 5-10 min	pH 5.5	S	Decreased
	Boiled anemic rabbit plasma	60-80% alcohol	pH 5.5	P	Unchanged
SMP	Boiled anemic rabbit plasma	TCA : 5°	pH 5.5	S	Unchanged
LB	Boiled anemic rabbit plasma	$HClO_4$: cold : 5.5 – 0.5 N		S, P	Unchanged (?)
G	Anemic horse serum	80% acetone		P, S	Unchanged (?) (Retic)
HPYE	Boiled anemic rabbit plasma	(80%-sat.) $(NH_4)_2SO_4$	pH 4.5	P	Unchanged (?)
BGKL HPYE RAC	Boiled anemic rabbit plasma	Dialysis		Dialysand	Unchanged(?)
MR	Anemic rabbit plasma	0.1 N HAc : 25° : Dialysis	pH 4.7	S, Dialysand	Increased

Fig. 2.

though with shorter boiling this loss may not be important. The alcohol precipitation of the activity leads to further purification, apparently without further loss of activity. Slaunwhite, Mirand, and Prentice find that cold trichloroacetic acid treatment leaves the activity in the supernatant, with little or no further loss of boiled activity.[4] For the remaining treatments--perchloric acid (Linman and Bethel[5]), acetone (Gley[6]), or ammonium sulfate (e.g., Hogson et al.[7])--some or all of the activity is in the precipitate and generally no clear statement is made regarding further activity loss. Most workers have reported that aqueous EPH activity is nondialyzable.[2,7,8]

In contrast to these results, our acetic acid–dialysis treatment leaves all the activity in the supernatant and the activity is (often) significantly increased. The details of this simple procedure are as follows (see Fig. 3). The anemic rabbit plasma is treated with an equal volume of 0.1 N acetic acid (to pH 4.7) for 24 hr at room temperature. Some precipitation (P-1) occurs, but the activity remains in the supernatant (S-1). This is then dialyzed for 24 to 36 hours against distilled water (with 2 or 3 water changings), leading to additional precipitation (P-2) and a supernatant fraction (S-2) containing all the activity and roughly half of the starting material by weight. Precipitates are collected by centrifugation for 10 min at $1200 \times g$. Several years ago we found that pH 4.75 (acetic acid–sodium acetate) was favorable for work with active urine proteins (activity remained soluble), and subsequently adapted this pH for the present work with plasma. Gordon et al.[9] systematically studied the preservation and recovery of activity from a kaolin column as a function of pH and concluded that 4.8 was optimum for adsorption. This is, however, not generally used as a working pH by other investigators in the field.

Activity Comparisons

We shall now consider comparisons of activity pertinent to our procedure. To date seven batches of plasma from rabbits with phenylhydrazine-induced anemia have been

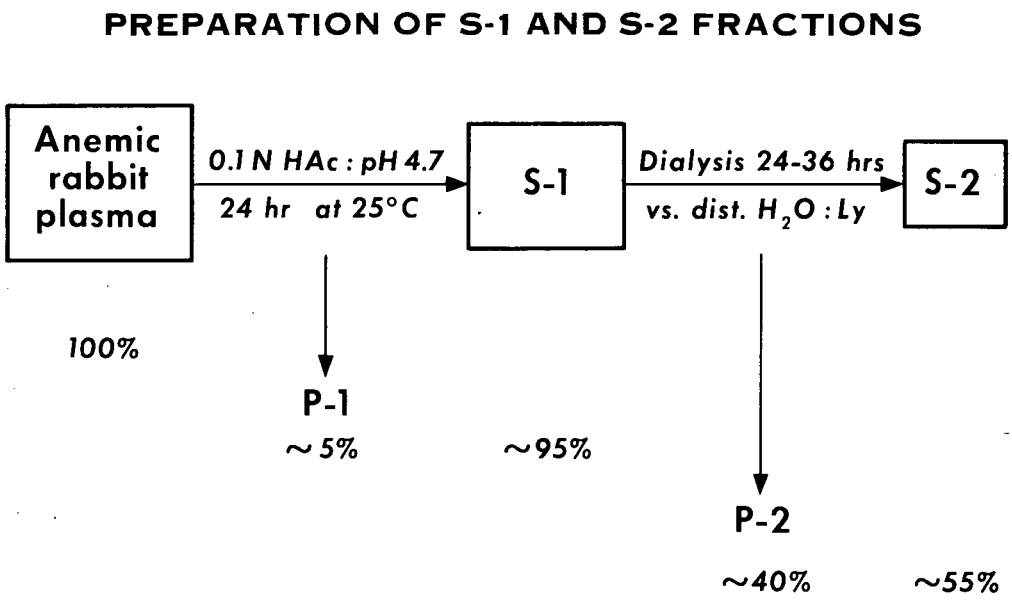

Fig. 3.

prepared by a slight modification of standard procedures (Appendix A) and assayed repeatedly (Appendix B). With few exceptions the activity of the starting plasma has been about 20% Fe^{59} uptake for a 2-ml daily injection schedule, with controls in the range 3 to 7%. A dose-response curve on recombined remnants of three pools is shown in Fig. 4, wherein the logarithm of the total volume of plasma injected (ml) per animal over the 3-day period is plotted against the Fe^{59} percent uptake. The number of animals per point

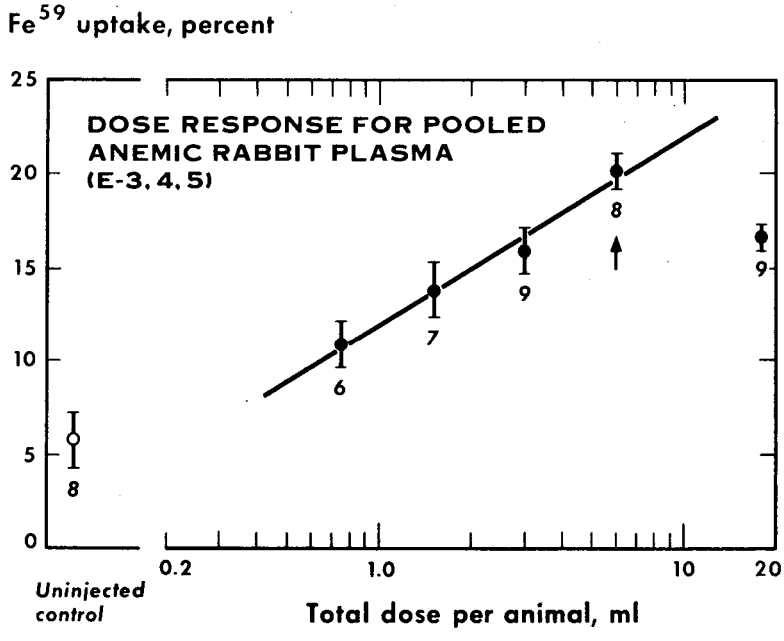

Fig. 4.

is shown underneath each point and the ranges indicated are for plus and minus the standard deviation of the mean for each point. The arrow indicates the usual injection schedule of 6 ml total, that is, 2 ml per animal per day. From comparison work in this and other laboratories, it appears that about the same response is obtained with this injection time schedule if the third injection is omitted entirely, that is, with a 4-ml total injection per animal. Except for the highest point, the exponential nature of the dose-response relation is evident. The low response for this very high point may result at least in part from difficulties of absorbing such large fluid volumes (6 ml per animal per day), and, in fact, unabsorbed fluid was later noted in the peritoneum of these animals. A somewhat similar saturation type of effect on a dose-response curve was recently noted by Reichlin and Harrington,[10] though these workers did not ascribe the effect to the same cause. The maximum Fe^{59} uptake we have ever obtained from our standard dose (2 ml daily) of native anemic rabbit plasma was 21.1%. Other workers generally do not report Fe^{59} activity on this same basis of starting plasma equivalent volume (especially for their later more highly refined fractions), but as well as we can tell from examination of the literature, and particularly by comparison with the results of the CalTech group (whose assay is most comparable), this is approximately the activity value to be expected from such a preparation.[11]

There are numerous references in the literature to the degree of loss of activity (more or less) after treatment (e.g. see the Gordon review[1]), but we have found none reporting increased Fe^{59} uptake per cc of starting plasma after any of the various physical or chemical treatments employed. Such an increase is apparently what we find, as indicated in Fig. 2. Although the value of this increase is not so uniformly predictable as is our starting activity, in almost every case we have tried there is either a slight or a major activity increase in our Fractions S-1 or S-2. Three examples of this are shown in Fig. 5. The native activity of each of the batches, which are representative ones prepared during the last 6 months, is seen to be close to 20%. Note that in each of these cases, after treatment the activity of the S-1 or S-2 fraction not only exceeds that of the

ACTIVITY IN ANEMIC RABBIT PLASMA FRACTIONS

	Fe^{59} uptake, percent				Factor
	Native	S-1	S-2	Uninjected controls	
E-1 7/22/60	18.5 ± 1.2 (5)	24.2 ± 1.8 (5)		6.2 ± 0.0 (5)	3.7 ×
E-4 9/16/60	19.3 ± 1.3 (5)		24.0 ± 1.1 (5)	6.2 ± 0.4 (10)	2.9 ×
E-7 1/2/61	21.1 ± 4.0 (5)	21.6 ± 0.3 (5)	28.2 ± 1.5 (5)	6.9 ± 0.6 (10)	5.1 ×

Fig. 5.

native plasma, but also exceeds the maximum (21.1% activity) that we have ever found in untreated anemic plasma prepared in this manner. The exact role of each step with respect to the activity increase is not yet clear, but experiments are under way to clarify this. The factors (listed in the last column) give the apparent activity increase after the treatment as determined from an extrapolation of the dose-response curve of Fig. 4. That is, because of the semilogarithmic nature of this dose response, an increase from 21.1 to 28.2% Fe^{59} uptake (taking batch E-7, for example) corresponds to a 5.1-fold increase in active material. The activity of the S-1 and S-2 fractions is well preserved when they are lyopholized or frozen, though there may be loss if they are stored for longer periods of time at room temperature.

We have also indicated in Fig. 6 some data regarding a patient, S. J., a 43-year-old male with secondary polycythemia (Appendix C). Of a considerable number of primary and secondary polycythemia plasmas tested during the past year, his was the only active one. He is one of the rare, highly active patients cited in the literature who is not severely anemic. His native plasma activity on 8/19/60 was 14.8%, compared with 6.6% control. An additional portion of the same batch of blood run a week later gave an almost identical result, 14.4% Fe^{59} uptake, and an S-1 fraction prepared at the same time showed the greatly increased value of 28.4%. Two months later another batch of this patient's plasma showed the much higher native activity of 35.7%. At this time a recombined supernatant-precipitate fraction, S-1 plus P-1, showed a 30.7% Fe^{59} uptake, which is slightly but not significantly lower than the native activity at that date. The failure of such a recombined supernatant-precipitate to show an activity increase is now under investigation. To be sure that this patient's activity was not transitory, a third batch taken 12/10/60 was assayed and also showed high activity, 25.2% iron uptake. The factor "24 ×" for the 8/26/60 experiment is the apparent increase in active material based on extrapolation of the rabbit plasma response curve (Fig. 4), which of course could not be expected to be necessarily valid in this case.

ACTIVITY IN SECONDARY POLYCYTHEMIA

Patient S.J.	Fe^{59} uptake, percent				Factor
	Native	S-1	S-1 + P-1	Uninjected controls	
8/19/60	14.8 ± 2.2 (4)			6.6 (5)	
8/26/60	14.4 ± 3.6 (4)	28.4 ± 1.2 (5)		5.3 (5)	(24 ×)
10/21/60	35.7 ± 2.4 (8)		30.7 ± 1.5 (8)	4.4 (10)	
12/10/60	25.2 ± 1.4 (5)			4.3 (6) (3.6 NRP)	

Fig. 6.

Activity for polycythemia patients has generally not been reported in terms of Fe^{59} uptake.[1, 13, 14, 15] Korst and co-workers have made such reports, but with the highest activity noted, 10% Fe^{59} uptake compared with controls of 6% (in nitrogen-mustard-treated rats).[16, 17]

Rationale of Treatments

What can one hope to accomplish from an initial treatment, preliminary to more sophisticated high purification and chemical characterization procedures? First, we wish to preserve biological activity, for obvious reasons. Secondly, we should like to avoid increasing the chemical heterogeneity of the active molecules or their total (inactive) chemical environment. This latter is of considerable help in simplifying the purification (that is, removing inactive materials) and in the ultimate chemical characterization of the activity itself. In other words, we wish mild handling procedures. Thirdly, an increase in specific activity is desirable. In later steps it is of course essential, by the very definition of purification. In Fig. 7 we have listed some common physical and chemical procedures, particularly as applied to proteins, in approximate order of relative chemical trauma. This list clearly is somewhat arbitrary and incompletely specified, and does not necessarily apply to a given biological activity which may be resistant to any or all of these, but still there is a certain rationale about this type of scheme.

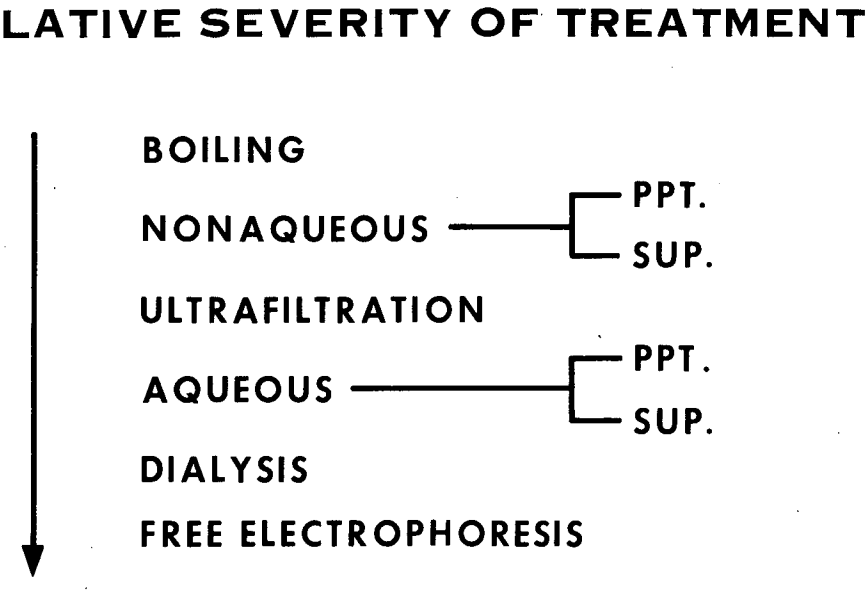

Fig. 7.

Boiling denatures most proteins, though EPH activity is quite resistant to boiling for short times. Nonaqueous treatments are generally more severe than aqueous, precipitations more so than extractions. Ultrafiltration appears to be approximately between aqueous and nonaqueous in severity. (A study of various preparative methods for serum

and urine proteins, including comparison of alcohol fractionation with ultrafiltration techniques, showed that vacuum ultrafiltration over about 1 week appears to cause the least change in the paper electrophoretic patterns of the proteins.[18]) Free-boundary electrophoresis is listed as an example of one of the mildest physical procedures. In such a scheme, then, what does our procedure offer as a first step?

1. Increase in activity. This is of course important from a preparative standpoint, and also bears on the question of multiplicity of active or inactivating substances.

2. Relative mildness. No boiling is involved; aqueous solution, supernatant extraction (not precipitation), and dialysis are used.

3. Modest purification (about 2x). This, of course, is a very minor degree of purification compared with what is ultimately necessary.

4. Improved properties for subsequent physical chemistry. This is noticed particularly for electrochromatography (see following section), and is under investigation for other procedures.

Other Physicochemical Aspects

We have some data on physicochemical changes associated with the preparation of Fractions S-1 and S-2, though the full story is still incomplete. A typical result for protein changes measured by analytical paper electrophoresis (bromphenol blue stain) is shown in Fig. 8. The most consistent change seen is the relative reduction in the alpha-1 and alpha-2 globulins. The separate alpha peaks sometimes disappear to the eye though the photoelectric scanner still gives some background (continuum) reading. Gamma globulins usually rise somewhat, with albumin and beta globulins remaining relatively the same. The glycoprotein changes (using periodic acid Schiff stain), qualitatively indicate a relative loss also of alpha-1 and alpha-2 globulins. This is of some interest in view of other literature reports as to the chemical nature of the hormone, shown in Fig. 9. (We have not included reports on nonprotein EPH.) White and Goldwasser used sheep rather than rabbit material.) It is to be noted that the three preparations by the different workers look like

PAPER ELECTROPHORESIS : TOTAL PROTEIN

Anemic rabbit plasma	Percent				
	Albumin	α_1	α_2	β	γ
E-7	41.6	10.0	6.8	15.7	25.8
E-7 (S-2)	42.5	—— 11.1 ——		16.4	29.7

Fig. 8.

CHEMICAL NATURE REPORTED FOR PLASMA EPH

White, Goldwasser (1960)	**α_1 - glycoprotein**
Lowy, Keighley, Borsook (1958)	**Protein : between alb - α_1, (α_2)**
Rambach, Alt, Cooper (1958)	**α_2 - glycoprotein**

Fig. 9.

at least three different chemical species.[19, 20, 8] Of course, defining a substance as e.g., an alpha-1 or alpha-2 globulin is really operational rather than precisely chemical. We believe that the differences reported may arise at least in part from differences in the various procedures themselves as discussed previously. Our results have suggested that the activity is increased when alpha-1 and alpha-2 total and glycoprotein content of the samples is decreased. Although this change was not in the direction we might have expected, we are not suggesting this is a contradiction to the reports shown in Fig. 9, for they are based on different and much more highly processed and purified EPH materials.

Electrochromatography

We have also undertaken some exploratory electrochromatography, both to evaluate its preparative role in the EPH problem and to further characterize our pretreatment fractions, as an aid to subsequent procedures such as free-boundary electrophoresis. Several years ago we were able to prepare high-specific-activity EPH at pH 4.7 from urine proteins in this manner (using an ultrafiltrate prepared by Dr. Donald Van Dyke), but the capacity of the method was low. Rambach, Alt, and Cooper (1957)[8] ran boiled plasma filtrates on a large electrochromatography apparatus at pH 8.6 for 7 days, but processed only about 200 mg, or approximately 30 mg of solids per day. Our S-2 fraction now appears to have greatly improved electrochromatographic properties, and we have recently processed 1300 mg per day in a smaller apparatus, in dilute acetic acid. Some problems still remain regarding loss of activity--we believe, to a certain extent, technical--but we are now planning higher-resolution experiments perhaps preceded by an additional aqueous extraction. With the preliminary low-resolution results we find, by spectrophotometric, pH, and paper electrophoretic analysis, considerable chemical fractionation of our S-2 material, with the activity migrating in an albumin-poor fraction.

Inhibitor

In considering the activity increase in our S-1 and S-2 fractions, one of the several explanations coming to mind is that in Precipitate 1 or Precipitate 2 (or by dialysis) we are removing a substance that interferes either with the Fe^{59} uptake process or directly with EPH itself--i.e., an "inhibitor." We have some other inconclusive results consistent with this idea, and further tests are under way. We find 1956 and 1960 literature references to an inhibitor,[21, 22] but from the information available these demon-

strations do not appear convincing. The very fact, however, that hypertransfusion stops erythropoietic activity in the usual RBC-forming organs[23, 24] is practically by definition a demonstration of inhibition of erythropoietic activity in a general sense. Other possible explanations for an activity increase per cc starting plasma include: (a) prevention of a possible routine loss of activity from anemic plasma, or (b) creation of a more active form of the EPH itself. Our best judgment based on our own reproducible 20% Fe^{59} starting activity, and comparisons with others using similar methods, argues against (a). We have no really direct evidence on (b).

Summary

1. Various "initial treatments" and physicochemical procedures in use with erythropoietically active blood are discussed, particularly with respect to their effects on the biological activity.

2. A simple acetic acid–dialysis pretreatment scheme is presented, which--unlike other treatments--actually leads to an increase of erythropoietic activity per cc starting anemic rabbit plasma. The possible relation of this result to the presence of an erythropoietic inhibitor is suggested.

3. Data are presented on the high erythropoietic activity (up to 35% Fe^{59} 17-hour uptake after three 2-ml injections in starved rats compared with 4.4% control uptake) and changes in this activity, for the plasma of a patient with secondary polycythemia.

4. Some preliminary observations on paper electrophoretic and electrochromatographic changes are presented.

5. Discrepancies between results from different laboratories are discussed in terms of the great diversity in sources of activity and in handling and assay procedures.

Acknowledgments

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Appendix

A. Production of Erythropoietically Active Plasma

New Zealand rabbits of both sexes, body weight about 3.0 kg, were injected with a 2.5% aqueous solution of phenylhydrazine hydrochloride (cp crystals, Baker & Adamson, Gen. Chem. Div., Allied Chem. & Dye) until the hemoglobin level in peripheral blood became just below 4.0 gr%. Injections were: 2 ml subcutan. on the first day, with subsequent 1 ml daily, until achieving this degree of anemia. This almost always required 6 to 8 days, though in one instance 11 to 17 days were required and this particular

batch showed lower than usual activity. Twenty-four hours later, animals under deep ether anesthesia were bled slowly from the vena cava with 100-ml syringes containing 10 ml of standard ammonium-potassium double oxalate anticoagulant. Each animal usually yielded more than 110 ml of blood. Blood was immediately centrifuged for 20 min at 4°C and about 1250×g. Plasma was decanted into chilled Erlenmeyer flasks and stored frozen until a batch of several hundred milliliters was collected.

B. EPH Assay in 150- to 200-g Female Long-Evans Rats (Fasted)

(See Plzak et al.[25] 0 hr: food removed; ip injections of test material at 24, 48, and 66 hours. (Liquid volume for each animal injection was 2 ml, except where greater than 2 ml plasma equivalent was to be injected.) 72 hours: ip, 0.1 ml, solution of $Fe^{59}Cl_3$, in normal homologous rat plasma. Each injection contained about 0.5 μC or 250,000 counts/min for the well-type scintillation counter. 89 hours: 2-ml blood samples withdrawn from vena cava from each rat for counting. Results are expressed as per cent of injected initial counts/min Fe^{59} recovered, assuming 5 % of body weight is blood.

C. Patient S. J.

This patient, with congenital heart disease, secondary polycythemia, and gout, has been treated under the direction of Dr. John H. Lawrence at the Donner Laboratory during the last 13 years. Treatment has included sulfamerazine, colchicine, P^{32}, and phlebotomy. Thorough cardiological investigation followed by heart catheterization revealed septal defect and pulmonary hypertension with predominant right-to-left shunt. Hematocrit during this period varied from 75 to 59%, and total red cell volume from 73.6 to 41.2 ml/kg.

			TRCV	
Date	Hct	Total Blood Volume	Total cc	ml/kg
1949	75	6606	4954	73.6
1951	71	6414	4554	73.1
1954	73	6604	4820	72.2
1955	75	6037	4528	67.0
	74	6682	4945	74.5
	62	4362	2704	41.2
1958	64	5468	3500	53.1
1959	61	6624	3716	58.8
1960	64.1	6439	3799	58.9
	59.0	9302	5051	77.7

Hemoglobin has varied from 19.6 to 10.8 gr%; RBC count from 13.3 to 6.2 million/mm^3, and peripheral reticulocyte counts from 3.2 to less than 0.1%. Except for an enlarged spleen, elevated uric acid level in serum (10.8 mg%), occasional cold, and subjective uneasiness, the other clinical and laboratory findings were normal.

Phlebotomy has been practiced regularly since 1954 and a total volume of 16 liters has been withdrawn in 28 bleedings. In 1949 the patient was given 3 millicuries of P^{32}, and at one other time x-ray therapy.

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WHOLE-BODY GAMMA-RAY SPECTROMETER

Thornton Sargent III

Construction of an enclosure within Donner Laboratory to house the whole-body counter and of an additional facility for other low-background counting has been completed. The rooms are partially subterranean and are protected by earthwork from the beam of the Crocker cyclotron, which produces a highly variable background in other parts of Donner Laboratory. Records obtained by means of continuous monitoring of the background level in these rooms were compared with those obtained by means of a simultaneously operating monitor in the area most affected by the cyclotron. Background radiation was found to fluctuate by a factor of 5 in the area affected by the cyclotron, whereas no detectable simultaneous fluctuation was observed in the new facilities. There, background is satisfactorily constant, as judged by the continuously recording ionization chamber. The background, however, was not especially low, since no special shielding was used in this determination.

Construction of the steel room was delayed because of difficulties encountered in procurement of steel. Surplus 6-inch armor plate from old naval vessels is now virtually impossible to obtain. If there is not an appreciable difference in radioactivity between old and new steel, cost factors may well dictate use of new steel. The metal finally used for the Donner steel room consisted of 6-, 3-, 2-, and 1-inch plates. The difficulty in cutting, cleaning, and rigging plate of this size and in erecting it in a relatively small space made the cost of construction very great. When this sum is added to the cost of the steel at surplus prices, the total cost was almost as great as if newly milled steel had been used.

Although the steel room has been completed, no measurements of background have been possible yet. The steel came from a variety of sources: two walls and the roof are of three layers of 2-inch plate milled in about 1955 for submarine construction at Mare Island Naval Shipyard. This material was obtained from the shipyard as surplus. Two walls are of 6-inch Class A armor plate (with the case hardening on the outside of the room). This had been ballistic test plate, supplied by the U. S. Naval Radiological Defense Laboratory and Hunters Point Naval Shipyard. Part of one wall is from a 6-inch bulkhead removed from the U.S.S. Chicago during alterations at Hunters Point. The floor consists of four pieces of Class B armor plate. Stocks intended for cyclotron shielding were traded for some of the Mare Island steel. Although this combination of steel presented problems in design and fabrication, it was all that was available. An opportunity to compare the different steels in terms of their content of radioactivity is a possible advantage. By placing a collimated sodium iodide crystal almost in contact with the center of each wall, it may be possible to detect differences in radioactivity if they exist.

The counting equipment intended for use with the steel room has been in operation for almost a year. It consists of a sodium iodide crystal, 4 inches in diameter by 4 inches thick, canned in electrolytic copper with a fused silica window, mounted in a yoke of #304 stainless steel. It was planned to use a Dumont Type K1730 lime glass 5-inch

itary. A literature survey revealed that although it is generally accepted that hemachromatosis is characterized in its early stages by excessive storage of iron in the body, the evidence for this is based on data with large errors.

The iron kinetics studies of this patient at Donner Laboratory by Dr. Pollycove and Dr. Richard A. Wetzel demonstrated that 75% of the iron received intravenously deposited in the red blood cells, and the remainder was stored, chiefly in the liver and marrow. The amount stored is about 2.5 times the normal amount. An oral-uptake study was undertaken. The patient received 50 μC of Fe^{59}. With 1-meter arc geometry and temporary partial shielding it was expected that the oral iron uptake could be determined accurately enough to gain some useful information about the disease. By using this geometry, it was found that the patient still had 14.3 μC of Fe^{59} remaining from the previous intravenous dose. One week after taking the oral dose, he had an additional 14.5 μC, which represented an absorption of 29%, almost three times the normal amount. The stool collections for the period between the oral administration and the whole-body determination were counted and compared with a standard. (It was found necessary to homogenize the specimens collected, as it was apparent that the activity was not evenly deposited, and the specimen was counted at 1 meter from the single crystal.) The stool count represented 69.5% of the oral dose, a very satisfactory agreement considering that the positioning error with this geometry was about 2%.

Iron kinetics and whole-body and stool radioactivities measured in a single patient give evidence that hemachromatosis in its early stages in this individual is characterized by absorption of iron and deposition in body stores at a rate almost three times that of the normal rate.

A variety of cases of exposure to internal radionuclides were studied with the temporary shielding arrangements. Body burdens ranging from negligible to zero were found for all these patients.

The first case involved a man who occupied a room where 2.2-yr Cf^{252} was used. Swipes in the room revealed that the isotope was present on ash trays and other objects, although not in amounts that represented great hazard. The potential exposure was oral or by inhalation, or both. A sample of this isotope was counted first in a Presdwood chest phantom. It is detected most readily by the gamma rays in the energy range 0.05 to 0.15 Mev resulting from the 3% spontaneous fission of this isotope. Within this temporary shielding, the subject was found to have no burden within the limits of uncertainty of about 0.017 μC i.e., within about 0.5 of the maximum permissible bone burden.

The next case involved three men who inhaled 129-day Tm^{170} in the hot cell of a near-by reactor installation. The maximum permissible burden for bone is 9 μC, for total body, 60 μC. A calibrated sample of the material was submitted also. Depending on the accuracy of calibration of the sample, the limit of detectability for this isotope is between 0.1 and 1.0 μC. Within this limit, no Tm^{170} was detected in the men, although one man had a slightly elevated count in the low-energy Compton region. This man has

photomultiplier without base, but the tube developed a crack around the lead wires and a photomultiplier from stock is now being used.

The crystal-photomultiplier-preamplifier is followed by an ultralinear nonoverloading amplifier with 0.5-μsec clipping, and a Penco 100-channel pulse-height analyzer. Data are recorded by a printer and a Mosely Autograf point plotter. This equipment has proved quite reliable and is relatively free from channel drift after a brief warm-up.

A larger sodium iodide crystal has been ordered. Multiple 4-by-4-inch crystals were considered. Advocates of the multiple-crystal array claim shorter counting times and greater independence of body size in K^{40} determinations; however, Miller et al.[1] have produced evidence that ability to detect small photopeaks in the presence of a Compton spectrum is much greater with a large crystal than with a small one. A cluster or other arrangement of small crystals would not improve matters, since it is the ratio of photopeak to Compton scattering that is critical in this case. Simply summing the counts from a number of small crystals does not change the ratio. High counts in the Compton region are especially troublesome when they obscure photopeaks in that region. Further, for multiple crystals, the requirement of a geometry that is completely reproducible, yet flexible, should presumably vary with patients of different body build. This introduces considerable complexity.

One of the primary uses of the Donner whole-body counter will be in studies of subjects who are accidentally exposed internally to radionuclides at the Radiation Laboratory. A wide variety of exotic and sometimes unexpected isotopes are often discovered in such cases, and it was felt that a single large sodium iodide crystal would best meet the demand for good gamma-ray resolution and lowest Compton levels. For rapid determinations of patients with an administered or natural dose of a known nuclide, sodium iodide crystals and 100-channel analyzers are an unnecessary expense, and for such patients a high-geometry plastic scintillator is being considered.

The 4-by-4-inch crystal installed with an unselected photomultiplier produced a resolution of 10% (expressed as full width at half-height of the Cs^{137} 0.662-Mev gamma photopeak). This crystal has been used in a variety of geometries with temporary and partial lead brick shields to study a number of accident cases, and patients with nuclides given as tracers.

The four clinic patients who were studied had been given large doses of Fe^{59} as tracers for iron kinetics studies by Dr. Myron Pollycove of this Laboratory. Three of them were counted for whole-body Fe^{59}. The stool collections for one week were counted also, to eliminate intestinal blood loss as a possible cause of anemia.

The fourth patient was referred by Oak Knoll Naval Hospital. He was a 23-year-old man who had developed jaundice from mild infectious hepatitis which rapidly cleared. Although his plasma iron was normal, a liver biopsy showed excessive iron deposition. His family history revealed that his father and an uncle had died of hemachromatosis and that two other uncles were presently suffering from it. It thus appeared that this patient was very likely in the early stages of development of this disease, which is considered hered-

had a long history in the field of atomic energy, and will be counted again when the counter is operating with the steel room.

Two accidents occurred involving small wounds in the hand with possible exposure to At^{211} and Pa^{231}. About 10^{-2} μC of the 8.0-yr daughter Bi^{207} was found on the tweezers causing the At^{211} wound, but none was found in the hand. The possibility of exposure in the Pa^{231} case was slight, and none was detected in the wound. A calibrated sample of Pa^{231} was supplied by a chemist working with it, but the gamma-ray spectrum was not that reported elsewhere for Pa^{231}. Since considerable amounts of this isotope are used in the Radiation Laboratory, a dependable standard is being prepared.

The necessity and value of good gamma-ray pulse-height resolution was demonstrated in two other cases. Two men inhaled dust from a sandpapering operation inside the chamber of the 90-inch cyclotron at the Livermore Laboratory. The dust was found to be radioactive, and pulse-height analysis at Livermore revealed a peak at 0.51 Mev, suggesting a positron emitter. The rest of the spectrum had virtually no structure. Pulse-height analysis with the 4-by-4-inch sodium iodide crystal at Donner Laboratory, however, showed eight photopeaks, which matched the spectrum reported for 77-day Co^{56}. The sample on which this was determined was a nose swipe from one of the men, and contained about 3×10^{-3} μC of Co^{56}. Counts of the men, with partial shielding and the crystal centered on the chest, showed a barely detectable burden, of the order of 0.02 μC. Although Handbook 69 does not list this isotope, the maximum total-body permissible level should be the same as that for Co^{58}, which is 30 μC. The Co^{56} is to be expected when iron in the cyclotron structure is bombarded by protons, by a p, n reaction with 92% abundant Fe^{56} in natural iron.

Another subject inhaled 33-day Rb^{84} from bombardment of krypton. He had no burden greater than 0.01 μC, and although a maximum permissible burden is not given for this isotope, that of 30 μC for Rb^{86} is a fair guide. Still another subject inhaled 36-hour Br^{82} and was found to have about 0.01 μC deposited in his chest, and perhaps 0.05 μC in his hair, even after five washings. The maximum permissible burden is 10 μC for the total body, and since the half-life is so short, this represented a very slight hazard.

Although no subject had more than a negligible burden, two patients, namely those exposed to Co^{56} and Br^{82}, had burdens which, had the complete whole-body counter been operative, might have presented us with an opportunity to study the half time in the body of these elements. With the whole-body counter, we will be able to gain much more complete information about deposition following accidents.

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