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ARGONNE CANCER RESEARCH HOSPITAL
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Semiannual Report to
THE ATOMIC ENERGY COMMISSION

MARCH 1966

LEON O. JACOBSON, M.D.
Editor

MARGOT DOYLE, Ph.D.
Associate Editor

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HIGH-DOSE HUMAN EXPOSURE FROM A 10-MeV LINEAR ACCELERATOR*

By

L. H. Lanzl, M. L. Rozenfeld, and A. R. Tarlov

The development of particle accelerators during the last decade has taken two directions. One has led to machines reaching tens of billions of electron volts but having modest average currents, the other to the development of units of large average current, with energies in the 10 MeV range. The health and safety aspects of the radiation environments of these two categories of machines differ widely. This paper deals with the dosimetry and other aspects of an accident involving an accelerator of the second variety.

In February, 1965, an industrial worker was accidentally exposed to radiations near and on the edge of a radiation beam consisting primarily of electrons, when he walked into the room in which a linear accelerator was in operation.

The accelerator is mounted so that 10.8 MeV electrons are directed downward after emerging into the air from a vacuum chamber at a height of 75 cm from the floor (Figure 1). The maximum peak electron beam current is 200 milliamperes, and the beam pulse length can be varied

Figure 1. Over-all view of variable-energy (4-12 MeV) microwave linear accelerator together with conveyor system. The beam is directed downward onto materials placed on the conveyor belt.

* This report is taken from a paper that was presented at the First Symposium on Accelerator Radiation Dosimetry and Experience, sponsored by the United States Atomic Energy Commission, at Brookhaven National Laboratory, Upton, Long Island, New York, on 3-5 November 1965.

from 2 to 10 microseconds. The accelerator is capable of being operated with single pulses or with repetition rates up to 180 pulses per second. The machine is provided with a scanning system for beam spreading which operates at a scanning rate of 5 cycles per second. The beam cross section at the exit window of the scanning chamber is 3 cm by 26 cm.

The beam spreads rapidly in its downward course due to its intrinsic angular distribution coupled with multiple scattering in the window and air. At a floor height of 46 cm, it strikes a conveyor belt whose function is to carry industrial products through the beam. A schematic view showing the accelerator in relation to the conveyor belt, radiation labyrinth, and service and control rooms is given in Figure 2.

Figure 2. Schematic drawing of floor plan showing accelerator room, access entryway through radiation labyrinth, service and control rooms.

Immediately after the accident, and also throughout a subsequent reconstruction of the event, dosimetric information was assembled to provide the physicians with information that might be useful in making an early prognosis of the extent of radiation damage to various parts of the injured man's body.

Figure 3 is a partial floor plan with isodose rate lines at the floor level for the beam current used in the routine production operation. The entrance through which the worker passed is on the lower left of the figure. The conveyor system runs across the top of the figure. The octagonal mold that the worker carried to the conveyor belt is shown as he left it. The dotted line indicates one axis of a coordinate system set up to facilitate recording of data on the placement of radiation detectors about the accelerator, and coincides with the direction of the beam scan over the conveyor.

Figure 3. Schematic drawing of floor plan in the vicinity of the accelerator proper, showing isodose lines at the floor level. The conveyor belt, with the octagonal mold in place, extends across the top of the figure. The vertical dotted line indicates a coordinate system coincident with the scanning beam location and direction over the conveyor. The rectangular block shown near the conveyor represents the position of a mechanical pump. Parts of the radiation labyrinth are shown in a crossed-line pattern. The location of a ladder (center), a television camera and a bank of lights (bottom) is also indicated.

The dose rate levels were measured by means of lithium fluoride thermoluminescent dosimeters calibrated at the equilibrium depth in a water-like phantom against a collimated beam of cobalt-60 gamma rays. These dosimeters, together with a Controls-for-Radiation reader, are very convenient since they function over several decades, and the reader can be switched to the proper decade range after heating the dosimeters, but before read out. An intercomparison at several points in the electron field and in the cobalt-60 gamma ray field was made with Sievert-type ionization chambers. It was found that the ratio of response in the electron and gamma radiation fields was the same for both types of dosimeters.

Figure 4 is a sketch of the accelerator, showing scanning chamber, and conveyor on the right. The left of the picture shows the location of a television camera and a bank of lights. The figure of the man does not indicate the location of the worker during irradiation, but shows scale only. The isodose rate curves in this figure were determined by thermoluminescent dosimeters in the range of 1000 rads per second and lower. The higher isodose rate levels around the exit end of the accelerator and conveyor were determined by cobalt-glass dosimetry. This method involves the change in optical density at 5300 angstroms following irradiation of the glass. These data were calibrated* by means of a cobalt-60 source whose output had previously been measured using chemical and ion chamber dosimetry. The calibration curve for cobalt glass is not

* This calibration was carried out by Keith Clark.

Figure 4. Isodose lines in a vertical plane as defined by the dotted line in Figure 3. The end of the accelerator, scanning chamber and conveyor are shown on the right. A television camera and a bank of lights are on the left. The silhouette of the man is shown for scaling purposes only.

quite linear. The value of net optical density change for one megarad is 0.34.

Figure 5 is a more detailed sketch of the equipment and of the isodose rate lines in the radiation field. The view is in a vertical plane normal to that shown in Figure 4. An edge-on view of the scanning chamber is shown in the center of the picture. The mold is in the position on the

Figure 5. Isodose lines in a vertical plane normal to the dotted line in Figure 3 and in the midline of the conveyor belt. The accelerator and the scanning coils are at the top. The scanning chamber is in the center. The cross-hatched lines indicate the conveyor. The mold as positioned by the worker is shown on the lower left.

lower left, where it was placed during the accident. The conveyor belt extends over the entire bottom of the picture. It is to be noted that the mold was placed on a brick which was directly on top of the conveyor. The worker's left hand grasped the mold on the left side in the dose region of 30 rads per second, and the right hand, on the right side, in the dose region of 10 K to 30 K rads per second. The mold was held with the thumb on top and the fingers curled underneath. Thus, the fingers received some protection from the mold. Of course, when the mold was released by the right hand, the fingers presumably swept through the fringe beam without the benefit of this shielding.

The dose distribution within the body is of prime importance. Such distributions depend on several parameters, such as the type of radiation, i.e., x-rays, gamma rays, electrons, neutrons, etc.; energies of the radiation; and the collimation of the radiation beam as contrasted with open sources with various degrees of isotropy.

To establish direction, degree of isotropy, and degree of penetration (i.e., energy) of the electrons, a simple circular phantom of unit density was used with Adlux film as a detector. This film was chosen because of its low sensitivity. The phantom was constructed of a stack of 8 cm diameter Masonite disks with circular pieces of film embedded among the disks. The results of the measurements are shown in Figure 6. The crescent shape of the blackening on all

Figure 6. The direction of the scattered electron beam at various locations in the vertical plane defined by the dotted line of Figure 3 is given by the arrows. The arrow pointing down on the right side of the sketch shows the direction of the primary beam as it leaves the accelerator and scanning chamber and strikes the conveyor belt. The numbers at each arrow location indicate the depth of the 50% dose point of the beam, measured using a tissue-equivalent phantom (see text).

the films indicates a high order of unidirectionality of the scattered electrons, the source of scatter being the conveyor. The direction (see Figure 6) of the scattered electrons at various locations is indicated by the arrows in a vertical plane as defined by the dotted line of Figure 3. The arrow pointing down on the right of the sketch shows the direction of the primary beam as it leaves the accelerator and scanning chamber and strikes the conveyor belt. The numbers at each arrow indicate the depth of the 50 per cent dose level of the beam.

The 50 per cent dose depth of the primary beam was determined by a rectangular unit-density phantom in which 3-mm-thick window glass was embedded in a plane parallel to the primary beam. The discoloration of the window glass served as the radiation detector. The extremely high output precluded the use of film in this primary beam study. The high absorption of the glass itself resulted in a 50 per cent dose depth of 3.6 cm, which is slightly less than the highest 50 per cent dose depth of 3.8 cm measured with film. It is interesting to note that the depth of penetration, averaging about 1.2 cm, does not differ much at any of the locations several feet away from the conveyor, even though the total scattering angle varies from 90 to 180°. The 50 per cent dose depth of 1.2 cm indicates an effective electron energy of about 2.5 MeV in the scattered beam.

Figure 7 shows the direction of the scattered electron beam and depth of the 50 per cent dose at various locations in the vertical plane normal to the dotted line of Figure 3 and at the midline of the conveyor belt. The arrow pointing downward in the lower center of the figure shows the direction of the primary beam as it leaves the scanning chamber. The conveyor belt and the mold position are shown at the bottom of the sketch.

Figure 7. Direction of the scattered electron beam at various locations in the vertical plane normal to the dotted line of Figure 3 and at the midline of the conveyor belt. The arrow pointing down in the lower center of the figure shows the direction of the primary beam as it leaves the scanning chamber. The conveyor belt and the mold position are shown at the bottom of the sketch.

The above measurements were made in the vicinity of the accelerator, without the presence of a whole-body phantom. These studies were followed by a determination of the location of the worker during the greatest exposure to his body. This location was then used in conjunction with body and extremity phantoms to measure the absorbed dose received by the worker in various areas.

An attempt was made to duplicate as closely as possible the actions of the worker when the accident occurred. The information used included statements of the worker himself and of his co-workers who were not eye witnesses; the location of equipment and facilities (especially the mold), the vacuum pump, the scanning chamber and shielding walls at the time of the accident; the location of radiation burns on the patient, and re-enactment sequences.¹ Detailed radiation field patterns were correlated with the radiation burns. These detailed patterns were determined by the radiation browning of 61 cm x 61 cm x 3 mm thick window glass placed at locations corresponding to the worker's right hand and forearm, right foot and right leg. Figure 8 is a photograph with two of these pieces of glass in place after irradiation. The upper part of the photograph shows the scanning chamber together with the browning of the glass in the region of the

Figure 8. Photograph of scanning chamber, top, conveyor with mold, center, together with window glass showing beam browning in the region of the right forearm position. At the bottom, the outline of the worker's foot position is shown on a second piece of radiation-browned glass.

right forearm. The conveyor belt is seen in the center of the picture. At the bottom, the outline of the foot position is visible on the browned glass pattern. It is to be noted that the toe region of the right foot was partially protected by the edge of the conveyor belt side support. This area of protection and foot location was directly verifiable by the location and extent of the actual radiation burns on the foot and leg. X-ray films could have been used in principle for some of these detailed pattern studies, but window glass has the advantage of not requiring development. (Darkroom facilities for film development were not immediately available, but a glass store did exist directly across the river from the scene of the accident.)

Figure 9 is a photograph of a person in the position of the injured worker. A rigid hetero-

geneous phantom² clothed in a laboratory coat and eye glasses was placed as nearly as possible in the trunk and head positions indicated in Figure 9.

Figure 9. Photograph of model in region of greatest radiation exposure. Note that the right hand is on the fringe of the direct beam and the right foot is slightly forward of the left foot.

A number of exposures of this phantom were made to determine dose levels on the surface and in the interior of the trunk and head. Figure 10 is a photograph of an exposed piece of Adlux

Figure 10. Photograph of Adlux film exposed in phantom to electrons at abdominal level. Note film blackening on edge of right front portion with little internal blackening.

film placed at the level of the abdomen. The darkened edge indicated a high surface exposure on the worker's front right side, with little radiation to the left rear portion of the body. It is especially important to determine radiation-free skin areas in cases where heterotopic skin grafts are indicated. The clear area in the center of films such as that illustrated by Figure 10 indicates that a minimal dose was received by the internal organs of the abdomen, thorax, and head.

Thermoluminescent dosimeters were also used in and on the phantom. To determine the time during which the worker was in the radiation area, a series of exposures was made with a personnel film badge and thermoluminescent dosimeters located on the phantom at the position of the worker's film badge during the accident. A plot of the dose versus the net optical density readings behind the open window, aluminum, and two thicknesses of silver filters is given in Figure 11. The net optical density readings of the accident exposure are shown as a series of four horizontal lines in the figure. Averaging these readings gives a dose to the film badge, i.e., to the skin, of 310 rads. This dose, together with the usual operating current of the accelerator as monitored, but not recorded, by the current pickup from an aluminum plate placed below the conveyor belt, indicates that the worker was in the radiation field for 8.5 seconds.

Figure 11. Calibration curve of personnel film badge. Net optical density is plotted against dose. The net optical density readings of the accident exposure are shown as a series of four short horizontal lines. An average of these readings gives a dose of 310 rads to the film badge, i.e., skin.

The dosimeter readings showed that the skin on the anterior and right lateral surfaces of the trunk of the body received doses in the range 245 to 325 rads. The interior of the body received doses ranging from 0.2 to 5 rad. These dose levels, arrived at by accident reconstruction, were consistent with estimates made immediately after the accident based on bremsstrahlung formation in the skin of the chest from a highly scattered electron beam. These early estimates had provided great confidence that the injured person would survive, since the blood-forming tissues would not have been impaired at this dosage level.

To establish the dose levels in the right foot, a paraffin foot phantom containing a skeleton was placed in a shoe at the position indicated in Figure 8. The toes on the foot received an esti-

mated 11,000 rads, while the ankle dose varied from 29,000 rads at the top surface to 300 rads at the base of the foot. These figures were determined by assuming the right foot was in the indicated position for 8.5 seconds.

A forearm and hand paraffin phantom containing a skeleton was also irradiated. This phantom did not articulate so that the position of the fingers was approximated and lacked some shielding by the mold. Dosimeters were placed at various parts on the surface of the hand and between the fingers. The dose gradient about the primary electron beam was high as shown earlier, giving an estimated dose to the thumb of 240,000 rads and to the little finger of 42,000 rads. These estimates are based on the assumption made from an interview with the worker, that the hand was in the beam for the same length of time as the trunk of the body, namely, 8.5 seconds. It is possible, however, that his hand was in the area for a shorter time. Therefore, one might view these dose figures as the upper limit for the hand exposure.

The doses received by various parts of the body are summarized in Table 1.

Table 1
DOSE DETERMINATION BASED ON PHANTOM MEASUREMENTS
FOR AN 8.5 SECOND EXPOSURE

Location	Dose in rads
Right hand, thumb	240,000
little finger	42,000
Right foot, ankle	29,000
toes	11,000
bottom of foot (arch)	300
Eyes, with glasses	43
Anterior and right surface of trunk, range	240 - 325
Posterior and left surface of trunk	1.4 - 6
Interior of trunk	0.22 - 4.9

The accident occurred in Rockford, Illinois, on 18 February 1965, at 3:15 p.m. By 6:00 p.m., the patient was in the University of Chicago Hospitals. At 7:00 p.m., erythema of the dorsal surface of the right hand, with greatest intensity on the thumb, was observed. The erythema also involved the wrist and the distal 20 per cent of the forearm, as well as the lateral aspect of the right leg from five inches below the patella to the ankle. There was neither pain nor motor nor sensory deficit.

Treatment was aimed at prevention of infection and control of pain. The most prominent clinical observations were related to development of swelling, erythema and blister formation, pain and loss of neurologic function, and eventually amputation.

At 11:00 a.m. on February 19 (Day 2), there was erythema over the entire hand and the distal third of the forearm. Swelling was most marked about the thumb, beginning at its base and extending backward to the mid-forearm. Later that day, the patient described a burning sensation at the wrist between the thumb and index finger. On the following day, he complained that his thumb felt cold. On Day 4, February 21, two bullous lesions, 3 x 4 cm and 2 x 2 cm, appeared on the dorsal surface of the right thumb, extending to the index finger. Medication was necessary

to control pain. Although movement of the fingers was limited by swelling, there appeared to be no neurological deficit on Day 5.

By Day 8, the swelling had extended to the axilla and involved the anterior axillary folds and anterior chest wall. All sensation in the thumb had vanished, except for pain on movement at the metacarpal phalangeal joint. Bullae formed over the thenar and hypothenar eminences and extended into the palm. The skin over the thenar eminence turned white and the tissue below had a bluish hue. The right leg continued to show only mild erythema laterally.

On Day 9, the bullous lesions over the base of the thumb and palm continued to enlarge and weep serous gelatinous material. The enlargement, but not the weeping, ceased by Day 11 (see Figures 12 and 13). Edema appeared to be on the decrease, but still extended to the axilla.

Figure 12. Right hand, 28 February 1965, 10:00 a.m., Day 11.

Figure 13. Right hand, 28 February 1965, 10:00 a.m., Day 11.

On Day 12, Dr. Sean Mullan performed a transcutaneous cervical electric chordotomy,³ selectively removing pain from the right upper extremity but leaving tactile sensation and proprioception intact. This procedure, developed by Dr. Mullan at the University of Chicago, results in temporary loss of pain sensation which subsequently returns in two to twelve weeks, depending on the magnitude of the current used and the duration of its application.

Approximately two weeks after the accident, the bullous lesions ruptured. Healthy granula-

tion tissue began to grow in from the edges of the lesions, and although it seemed certain that the thumb would have to be amputated, there was hope that the hand and forearm could be saved. This was prior to collection and analysis of the extensive information on dosimetry. However, over the next few months progressive necrosis of the deep structures, muscle, tendon, nerve, and bone, slowly developed (see Figures 14 and 15 [Day 66]). Vascularity underwent complete deterioration. Infection with pyocyanous organisms supervened. Amputation just above the elbow was performed on 6 July, 138 days after exposure.

Figure 14. Right hand, 27 May 1965, Day 96.

Figure 15. Right hand, 27 May 1965, Day 96.

The delayed tissue response and the necrosis occurring after radiation injury are well illustrated by the course of events in the right foot and leg. Erythema of the anterolateral surface of the leg was present on the day of exposure, but remained stationary for twenty days before the skin broke down. Similarly, the foot appeared completely normal until Day 25, when small vesicles appeared about the nail beds. Generalized erythema of the foot appeared on Day 30, with

breakdown of the skin on Day 35 (see Figure 16, Day 70). Neuromuscular function was intact. Severe pain was treated effectively with another cervical chordotomy aimed at the pain fibers from the lower extremity. After three months of observation, surgical debridement was begun. Continuous discouragement was experienced as tissues that appeared to be viable one week became necrotic the next. Slow, but progressive loss of structure and loss of function of the deeper tissues followed, necessitating amputation just above the knee on August 24th, six months after exposure.

Figure 16. Right foot, 28 April 1965, Day 70.

The low radiation dose to the trunk of the body, which had been determined by dosimetric measurements, was confirmed by the clinical fact that the absolute lymphocyte count, normal on admission, remained normal throughout the entire illness. An early and sustained polymorphonuclear leukocytosis was relatively constant, with a total white count of approximately 18,000 per cubic milliliter. Anemia, thought to be due to chronic and severe inflammation, developed after two months and was sustained.

This patient underwent six months of severely debilitating illness, which culminated in double amputation. Much of this illness could have been averted by early amputation. Judgments in that regard might be facilitated in some future accidents 1) if it were possible to establish levels of exposure promptly, with a small range of uncertainty, and 2) if experiments in primates relating dosimetry to eventual tissue damage were available.

The information needed in this situation was not available in the extensive literature on serious radiation accidents. Some of this literature involves mixed neutron and gamma radiations of rather uniform distribution over the entire body.^{4,5} Other reports have dealt with radiations such as low-energy x-rays, or electrons with high-gradient distributions which lead to substantial doses to the surfaces of the extremities.⁶⁻⁸ To a limited extent, these latter reports deal

with accidents similar to that described here, but at lower dose levels and with a somewhat less penetrating radiation field to the extremities.

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We wish to thank the linear accelerator personnel and their firm's officials for their most cooperative help and advice in this study.

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DETERMINATION OF ABSORBED DOSE FOR ELECTRON BEAM THERAPY*

By

L. H. Lanzl, L. S. Skaggs, and M. L. Rozenfeld

Theoretically, if the spectrum and intensity of an incoming radiation beam, and the absorption properties of a medium are known, the absorbed dose rate can be determined. In most cases, especially in clinical situations, this is an impractical way of determining absorbed dose for x- and γ -ray beams, chiefly because of the complicated calculations that are involved. This type of calculation must include several modes of absorption of the primary beam, the mechanisms and locations of absorptions, and the deposition sites of the secondary radiations.

Sometimes the method is impractical even for electron beams, especially if beam broadening is accomplished by scattering foils followed by collimation. The use of scattering foils, particularly of thick foils, introduces energy degradation of the primary beam as well as rather complicated angular and radial intensity distributions. For a scanning beam of electrons, such as that used in the Argonne Cancer Research Hospital, where the beam is highly monoenergetic and the collimation is extremely good, such determinations of absorbed dose are practical.

The apparatus employed for this work includes an electron source and its beam deflecting and scanning device. The electron source is a traveling-wave linear accelerator whose electron energy can be controlled to give a sharp spectrum with a peak of from 5 to 50 MeV.¹ After leaving the accelerator, the electrons enter the magnetic deflection and scanning apparatus.²

Figure 1 is a schematic drawing of the apparatus. The beam enters the first magnet from point A. This magnet has a positional focus in the plane of the diagram at a point midway between the first and second magnets (point C). An energy selecting aperture is placed at this midpoint. The separation of the aperture leaves determines the per cent spread of energy that passes through. It is at this point, therefore, that the electron spectrum is determined. In actual use, the width of the energy window amounts to $\pm 1/4$ per cent of the energy. Since the aperture is placed at the point of a positional focus, the spectrum is extremely sharp on both edges, but may not always be flat, depending on the tuning of the accelerator. The percentage spread is so small, however, that it can be regarded as flat.

After leaving the aperture, the analyzed beam enters a second magnet, which gives a linear offset of 71 centimeters from the original beam path. The electrons then enter a third magnet, which bends the beam through 90° . This third magnet is capable of being moved so as to provide a scanning beam. The beam exits at point D.

Figure 2 demonstrates this apparatus in use for therapy. The electron beam is enclosed in a vacuum system until it emerges through the vacuum window after passing through the third magnet. A transmission ionization chamber is placed directly outside the window.

Figure 3 is a close-up view of the transmission ionization chamber, a template that controls the area over which the beam scans, and a retractable pointer for patient lineup.

*This report was presented at an International Meeting on Physical and Clinical Dosimetry of Fast Electrons, at the Instituto di Radiologia, Policlinico Umberto I, Roma, Italia, on September 28, 1965.

Figure 1. The deflecting and scanning unit includes a triple magnet system, counterweight, rotating shield and treatment cot. The electron beam striking the patient is contained within a vacuum chamber which is not shown in this drawing. The inset on the right side illustrates scanning over a single portal field.

Figure 2. Patient treatment area showing the final scanning magnet (top) together with the vacuum chamber and transmission ionization chamber (center) at the beam exit.

Figure 3. Close-up view of final magnet, transmission ionization chamber, and retractable pointer for patient lineup (center). The template that controls the area over which the beam scans is shown on the upper right of the photograph.

Figure 4 shows the ion chamber with the lineup device retracted. The ion chamber is used to determine the beam current and is shown in schematic detail in Figure 5.

The cross-sectional pattern of the electron beam at the exit port of the transmission ion

Figure 4. Ion chamber is viewed with the lineup device retracted.

Figure 5. Schematic drawing showing construction details of the transmission ionization chamber.

Figure 6. The cross-sectional pattern of the electron beam at the ion chamber. The shadow pattern of temporary cross wires is visible.

chamber is indicated by the photographic film shown in Figure 6. The ion chamber, which measures the entire beam that strikes the absorbing medium, is composed of two high-voltage electrodes with a central collection foil. The geometry of two high-voltage plates insures by electrical shielding a precisely determined path length through which the beam travels within the collecting field of the chamber. The ion chamber is also equipped with a safety shell which automatically turns off the beam and the scanning motion when it is accidentally hit. Beside the collection and voltage foils, which are composed of metallic-coated Mylar film, there are two thin aluminum windows, which provide a moisture and gas barrier. The current calibration, which is of great importance, is carried out with a Faraday cup.³

The Faraday cup, shown schematically in Figure 7, is composed in part of a low-Z material (graphite) in the region where the electrons lose their energy. This serves to minimize bremsstrahlung production. The graphite block is pierced axially by a one-inch diameter hole which penetrates quite deeply, thus reducing any loss of electrons by backward scattering. The graphite is followed by a thick lead piece which reduces the bremsstrahlung produced in the graphite so that the bremsstrahlung beam emerging from the cup is minimal. Thus the lead, in reducing the bremsstrahlung also reduces the number of Compton electrons produced at the interface of the cup and the vacuum chamber. A measure of the Compton electrons escaping from the cup assembly is obtained by means of an insulated graphite block behind the lead. At the front of the cup an insulated alignment ring serves several functions assuring accuracy.

Figure 7. Cross-sectional drawing of the Faraday cup (right) together with front view (left).

Many detailed checks must be made to provide an absolute measurement of the electron current of a high-energy beam. A few of these are discussed below.

To avoid the collection or loss of charge by ionized air surrounding the cup, it is placed in an evacuated tank. The vacuum window can be a source of secondary electrons when the beam passes through it. Since these secondary electrons are not a part of the primary beam, the number that enter the cup must be evaluated and correction made. In these particular measurements, the beam passes through a number of foils, and becomes slightly divergent. The amount of the beam that does not enter the cup is evaluated by means of the alignment ring, whose inner diameter equals that of the hole in the cup. The number of electrons striking the ring, as well as the number of electrons striking the cup, are measured under the same operating conditions as a function of added scatterer. By extrapolation to the thickness of the inherent scattering material, which is 0.42 mm of aluminum, one obtains a correction factor for the total current. A graph of Faraday cup collection current versus thickness of added scatterer is given in Figure 8. The data are plotted for scatterer thicknesses beyond those strictly needed for these measurements. There is, however, a rapid fall-off of cup current versus added scatterer.

In addition, measurements have been made subjecting the cup and ring to various voltages and pressures, and with various magnetic fields of the electromagnet placed at the opening of the cup. Analysis of these measurements gives an accuracy of the absolute current measurement of the order of 1 per cent at 20 MeV.

Figure 9 is a photograph of the Faraday cup in position in front of the beam deflecting and scanning mechanism. The vacuum system, with a forepump, diffusion pump, and trap, is included in the picture.

The next step in determining the absorbed dose deals with the absorption properties of the medium under consideration, here taken to be a water-equivalent material. Figure 10 is a typical central axis depth dose for a field size of 4 x 4 cm and an electron energy of 20 MeV. The data for this figure were derived from film. The general characteristics agree with data from foil scattering techniques for beam broadening.

The absorbed dose depends on the instantaneous rather than on the integrated current with the beam scanning technique. The technique involves covering the area to be scanned with a pen-

Figure 8. Plot of Faraday cup collection current, cup and ring, versus thickness of added aluminum scatterer.

Figure 9. Photograph of Faraday cup placed in front of the final scanning magnet of the linear accelerator deflector system.

cil beam of electrons. The beam is guided by the magnet system, which moves continuously in a line in one direction, and is then displaced orthogonally in a discrete step before returning on a parallel path.² The area covered by the beam per unit time is determined by the velocity of scan, which is 1.13 cm/sec at 61 cm, the standard treatment distance. The scan spacing is 1/2 cm, so that the area covered per unit time is $0.565 \frac{\text{cm}^2}{\text{sec}}$.

Figure 10. Central axis depth dose distribution.

To establish the intensity of the beam, a knowledge of the electron fluence is necessary. With an absolute current of 8.8×10^{-10} amperes, the electron fluence is:

$$\begin{aligned}
 J \frac{\text{electrons}}{\text{cm}^2} &= \frac{8.8 \times 10^{-10} \text{ coulombs/sec}}{0.565 \text{ cm}^2/\text{sec}} \times 6.25 \times 10^{18} \frac{\text{electrons}}{\text{coulomb}} \\
 &= 9.73 \times 10^9 \frac{\text{electrons}}{\text{cm}^2}
 \end{aligned} \tag{1}$$

passing through the patient's skin (or water phantom surface).

The absorbed dose (D) is given by:

$$D = J \frac{dE}{dx} \tag{2}$$

where $\frac{dE}{dx} = \frac{\text{energy absorbed}}{\text{unit thickness}}$ for each electron.

The quantity $\frac{dE}{dx}$ has been calculated under several conditions that are pertinent here. Near the surface of water, i.e., one millimeter depth in water, Sternheimer's⁴ formulation of restricted collision loss, which contains the density or polarization correction, was used. In particular, Equation 50 of Reference 4 used to calculate the loss to all secondary electrons up to 0.4 MeV, gives a loss:

$$\left. \frac{dE}{dx} \right)_{T_0 = 0.4 \text{ MeV}} = 1.754 \text{ MeV/cm} \tag{3}$$

The upper cut-off energy (T_0) of 0.4 MeV was chosen since this corresponds to an electron

range of one millimeter in water; i.e., electron equilibrium has been reached at one millimeter depth by all secondaries up to 0.4 MeV.

On the other hand, secondary electrons above 0.4 MeV will not have reached equilibrium at this depth, even though they too, contribute to the loss in the first millimeter. A calculation that takes into account the number of secondary electrons produced from 0.4 MeV to 10 MeV and the variation of collision loss with energy, shows an additional loss of 0.0358 MeV/cm. Secondary electrons are not taken into account above 10 MeV, since by convention, in an electron collision of a 20 MeV electron with another of a different energy, the electron with the lower energy is considered to be the secondary one. It is correct to do this because electrons are indistinguishable particles. Thus, the total loss per electron at one millimeter depth becomes:

$$\left(\frac{dE}{dx}\right)_{T_0 = 0.4 \text{ MeV}} + \left(\frac{dE}{dx}\right)_{\text{From } 0.4 \text{ to } 10.0 \text{ MeV}} = 1.754 \text{ MeV/cm} + 0.0358 \text{ MeV/cm} = 1.79 \text{ MeV/cm} \quad (4)$$

Using this loss figure, the electron fluence 9.73×10^9 electrons/cm² from Equation (1), and appropriate conversion factors, one obtains:

$$D = J \frac{dE}{dx} = 279 \text{ rads} \quad (5)$$

for the absorbed dose at one millimeter depth in water.

A further possible contribution to the absorbed dose at the one millimeter depth is that due to bremsstrahlung. Calculation of the total energy lost to bremsstrahlung by the slowing down of a 20 MeV electron gives 1.72 MeV. On the other hand, bremsstrahlung is produced throughout the electron range of the electrons, proceeds in the forward direction, and is deeply penetrating. Thus, its energy deposition is negligible at one millimeter.

Sternheimer's Equation (49)⁴ is used to calculate energy loss in the depth of the water because this equation takes into account all secondaries. Considering a primary energy electron of 20 MeV and secondaries up to 10 MeV, the resultant loss is:

$$\left(\frac{dE}{dx}\right)_{\text{depth}} = 2.066 \text{ MeV/cm} \quad (6)$$

It is demonstrated below that this loss figure represents the energy deposited at 2.5 cm even though secondary electron equilibrium has not been fully attained. Let us consider a depth in water at 2.5 centimeters and a maximum secondary electron energy of 10 MeV. If one uses the simplified model of all these electrons losing energy at a space rate of 2 MeV/cm up to 2.5 centimeters, equilibrium conditions will have been reached for all secondary electrons 0 to 5 MeV. Then if one calculates the number of secondary electrons with energy from 5 to 10 MeV that would cross the space from 2.5 to 2.6 cm if equilibrium had been obtained, it can be determined that these electrons would lose energy in this space at a rate of 0.0274 MeV/cm per primary electron. This represents a maximum departure from the figure of 2.066 MeV/cm due to failure to reach equilibrium. Subtracting this from (6), one obtains a minimum loss at 2.039 MeV/cm. This calculation assumes no straggling of electrons and no removal of primary electrons by bremsstrahlung or other secondary processes.

On the other hand, one can expect a slight increase of energy loss due to change of direction from multiple scattering. On the basis of a rough calculation, this increase would amount

to about 1.5 per cent. Thus the above two corrections approximately compensate for each other.

For the purpose of this paper, therefore, a compromise value of 2.066 MeV/cm is used for the loss at 2.5 cm depth and could be used at the maximum of ionization of the build-up curve (see Figure 10).

At 2.5 cm, the beam will be depleted by bremsstrahlung loss. Calculating the approximate number of electrons thus removed from the beam, we get a reduction of the beam current by 1/2 per cent, i.e., a correction factor of 0.995 for the electron fluence.

Using the value of $\frac{dE}{dx} = 2.066$ MeV/cm from (6), the value of $J = 9.73 \times 10^9$ electrons/cm² from (1) corrected for primary electron loss, 0.995, and appropriate conversion factors, the absorbed dose at 2.5 centimeter depth is determined to be:

$$D = J \frac{dE}{dx} = 320 \text{ rads} \quad (7)$$

The ratio of (5) and (7) gives a ratio of surface to plateau dose of

$$279/320 = 87.2 \text{ per cent.} \quad (8)$$

Our photographic film data gave a value of 89 per cent. Other workers obtain values from about 85 to 88 per cent at 20 MeV. The agreement is within the accuracy range of the film dosimeter. To go much further into the depth would increase the uncertainty in the calculation because of the mounting importance of bremsstrahlung and multiple scattering.

Unfortunately, there is as yet no simple dosimeter for electrons that a standards laboratory, such as the National Bureau of Standards, will calibrate. In the past, various individuals have exposed x-ray thimble chambers of the Victoreen type to electrons and used the resultant readings for patient dosimetry. For a particular chamber on hand at the Argonne Cancer Research Hospital, the reading for a thimble placed at a 2.5 cm depth in a water-like substance was, for a primary electron energy of 20 MeV and a primary beam current flux density of 8.8×10^{-10} amperes:

- 1) 312 when multiplied by a National Bureau of Standards calibration factor for 1 mm Cu HVL;
- 2) 349 when multiplied by a National Bureau of Standards calibration factor for cobalt-60 gamma rays.

These numbers are not accompanied by the unit roentgen, because it is not defined for electrons. Even though the numbers straddle the value of 320 rads for the in-depth determination, x-ray thimble chambers should be used with caution for electron measurements because they were designed for other purposes.

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650965-2

THIN FOIL CALORIMETER FOR ELECTRON BEAM DOSIMETRY*

By

L. S. Skaggs

The use of ion chambers for dosimetry of high density pulsed electron beams has frequently encountered serious non-linearity with dose due to the loss of collection efficiency resulting from recombination of ions. A new calorimetric method of measuring electron beam dose rate has been developed at the Argonne Cancer Research Hospital¹ that is independent of the pulsed nature of the beam when the pulse rate is above a few pulses per second. In this dosimeter the beam passes through a very thin metallic foil, normally within the vacuum system of the accelerator, releasing a small amount of energy as heat in the foil due to electron collision losses. Figure 1 shows a schematic diagram of the arrangement. Heat is conducted from the foil through its supporting "heat leaks" to the reference system so that the foil quickly comes to an equilibrium temperature that is proportional to the electron beam current. A thermocouple is used to measure the temperature difference between the foil and the reference system.

Figure 1. Schematic diagram of thin-foil calorimetric dosimeter for electron beams.

The rate at which energy is dissipated in the foil by a beam of i amperes is

$$\frac{dQ}{dt} = \frac{1}{4.18} \delta \frac{dE}{dx} \cdot i$$

where Q = quantity of heat, calories

δ = thickness of foil, cm

$\frac{dE}{dx}$ = rate of electron energy loss in the foil, electron volts per cm

Moreover, the rate at which energy is lost by the foil when the temperature rise is so small that radiation losses may be neglected is

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$$-\frac{dQ}{dt} = \frac{a}{\lambda} KV$$

where a = cross-sectional area of the heat leak, cm^2
 λ = length of the heat leak, cm
 K = coefficient of thermal conductivity of the heat leak, calories per $\text{cm}^\circ\text{C sec}$
 V = temperature difference between foil and reference system, $^\circ\text{C}$

In the steady state, when the rate of heat loss is equal to the rate of heat gain, the temperature difference will be designated V_{max} and

$$i = 4.18 \frac{a}{\delta \lambda} \frac{K}{dE/dx} V_{\text{max}}$$

If foil and heat leak have the same thickness and the width of the heat leak is w (cm), then

$$i = 4.18 \frac{w}{\lambda} \frac{K}{dE/dx} V_{\text{max}}$$

Thus the beam current is simply related to the steady state temperature difference by fundamental constants of the foil material and the width and length of the heat leak.

If a beam that will eventually produce the steady state temperature V_{max} is suddenly established through the foil, it may be shown that the temperature difference V will be the following function of time, t

$$V = V_{\text{max}} (1 - e^{-t/T})$$

where T is the time constant. It varies directly as the heat capacity of the foil and inversely as the conductivity of the heat leak. If

r = effective radius of the foil, cm
 C = specific heat of the foil material, calories per g°C
 ρ = density of foil, grams per cm^3

then it is easily shown that the time constant T has the following form:

$$T = \frac{4.18\pi r^2 C}{\frac{1}{\rho} \frac{dE}{dx}} \cdot \frac{V_{\text{max}}}{i}$$

Since V_{max}/i is a measure of the sensitivity of the foil, it is seen that for a given material and radius of the foil, the time constant can only be reduced at the expense of sensitivity. For such a foil, where the thickness of the heat leak is the same as that of the foil, the sensitivity and time constant are adjusted by variation of the length, λ , and width, w , of the heat leak. Since the thickness, δ , does not enter explicitly into the equations for sensitivity and time constant of the uniform thickness calorimeter, the foil may be as thin as mechanical considerations permit. Thus the perturbation of the beam may be reduced to a minimum.

Now consider the quantity $\frac{1}{\rho} \frac{dE}{dx}$, which measures the energy loss of high energy electrons in a medium. This loss will be almost entirely due to ionization processes, since bremsstrahlung and nuclear processes will be rare for a thin foil and even then, most of the energy loss will escape the foil. Ionization energy loss of a high energy electron in a medium includes losses to secondary electrons or delta rays with energy up to one-half the energy of the primary electron.

The high energy secondaries will escape from the foil and dissipate only a small fraction of their energy in the foil. If, in calculating $\frac{1}{\rho} \frac{dE}{dx}$, the concept of restricted energy loss is used to include all transfers of energy up to a fixed quantity Δ by the primary, then any contribution of energy loss to the foil by secondaries of energy greater than Δ will be neglected. Partially balancing this will be the inclusion of secondaries below the energy Δ that escape from the foil with some of their energy, or are produced so close to the surface of the foil that they escape with most of their energy. (For the relativistic primary electrons treated here, most secondary processes occur in the forward direction.) Thus the value of $\frac{1}{\rho} \frac{dE}{dx}$ for the foil may be calculated with the proper choice of Δ . It will be shown that the energy loss is not very sensitive to the value of Δ and that the response of the calorimeter with primary beam energy can be calculated with good precision in the relativistic region.

The formulation of Berger and Seltzer² will be used for restricted energy loss in which $\frac{1}{\rho} \frac{dE}{dx}$ is replaced by $L(r, \Delta)$ where r is the energy of the primary electron in units of mc^2 and all collision energy transfers up to Δ (mc^2) are included.

$$\text{Then } L(r, \Delta) = \frac{2\pi N r_o^2 mc^2}{\beta^2} \frac{Z}{A} \cdot \left\{ \ln \left[\frac{2(r+2)}{(I/mc^2)^2} \right] + F(r, \Delta) - \delta \right\}$$

$$\text{where } F(r, \Delta) = -1 - \beta^2 + \ln[(r - \Delta)\Delta] + \frac{r}{r - \Delta} + \frac{\frac{\Delta^2}{2} + (2r + 1) \ln(1 - \frac{\Delta}{r})}{(r + 1)^2}$$

which for $\Delta \ll r$, simplifies to

$$F(r, \Delta) = -\beta^2 + \ln(r\Delta)$$

Also

- N = Avogadro's number
- r_o = Classical electron radius
- m = Electron mass
- c = Velocity of light
- β = Primary electron velocity in c units
- Z = Atomic number of absorber
- A = Atomic weight of absorber
- I = Mean excitation energy of absorber

The quantity δ is now the polarization or density effect correction in which the formulation of Sternheimer³ will be used:

$$\delta = \begin{cases} \ln r(r+2) + C + a(X_1 - X)^n, & X_o \leq X < X_1 \\ \ln r(r+2) + C, & X \geq X_1 \end{cases}$$

where now

$$X = \frac{0.4343}{2} \ln r(r+2)$$

and for copper²

$$C = -4.43; a = 0.109; n = 3.39$$

$$X_0 = 0.2; X_1 = 3.0; I = 323 \text{ eV}$$

$$2\pi N r_0^2 mc^2 Z/A = 0.0701 \text{ MeV/g/cm}^2$$

The second form of the density effect equation gives the value in the region of the high energy limit (Fermi plateau) where, for copper, $X \geq 3.0$ ($r \geq 999 mc^2 = 510 \text{ MeV}$). Substituting for δ and $F(r, \Delta)$ in the original equation for $L(r, \Delta)$ yields

$$L(r, \Delta) = 0.0701 \left\{ \ln \frac{2\Delta}{(I/mc^2)^2} - 1 - C \right\},$$

since $\beta^2 = 1$. It may be seen that all terms in r have disappeared and the energy loss depends only on constants of the absorbing material and the secondary energy cut-off limit Δ . Furthermore the dependence on Δ is logarithmic. Substituting values for copper for the remaining terms yields

$$L(r, \Delta) = 0.0701 (18.86 + \ln \Delta)$$

If the limit Δ is chosen equal to $0.2 mc^2$, which corresponds to secondary electrons having a range in copper of $2.5 \cdot 10^{-3} \text{ cm}$, then $\ln \Delta = -1.61$ and

$$\begin{aligned} L(r, \Delta) &= 0.0701 \cdot 17.25 \\ &= 1.21 \text{ MeV/g/cm}^2 \end{aligned}$$

For comparison, the unrestricted energy loss in copper of $999 mc^2$ electrons is 1.79 MeV/g/cm^2 .

If the first form of the equation for δ along with that for $F(r, \Delta)$ is substituted in the original equation for $L(r, \Delta)$, then for copper

$$L(r, \Delta) = \frac{0.0701}{\beta^2} \left\{ \ln \frac{2\Delta}{(I/mc^2)^2} - \beta^2 - C - a(X_1 - X)^n \right\}, \quad X_0 \leq X < X_1$$

If the lower limit of r to be considered is ten, then $\beta^2 = 1$ and

$$L(r, \Delta) = 0.0701 \left\{ 18.86 + \ln \Delta - 0.109 (3.0 - X)^{3.39} \right\}$$

The energy loss to the foil of an electron in this range is seen to depend on the primary energy only to the extent of the logarithmic term X . The ratio of restricted energy loss of an electron in this range to that of an electron in the high energy limit (Fermi plateau) may now be expressed as

$$\begin{aligned} \frac{L(r, \Delta)}{L(\infty, \Delta)} &= 1 - \frac{0.109 (3.0 - X)^{3.39}}{18.86 + \ln \Delta} \\ &= 1 - \frac{0.109 (3.0 - X)^{3.39}}{17.25} \end{aligned}$$

for the previously assumed $\Delta = 0.2 mc^2$.

Figure 2 is a plot of the above ratio. The relative energy loss is only 6.2 per cent below that of the high energy limit at 5 MeV and rises rapidly toward the limiting value as primary energy increases. Furthermore, the next to last equation shows that Δ enters only as a small

Figure 2. Energy sensitivity of the calorimeter expressed as a ratio of response at a given electron energy to the response at very high energies.

additive logarithmic term in the denominator. The result is then not very sensitive to the choice of Δ and, in fact, decreasing Δ by a factor of ten increases the ratio by less than one per cent at 5 MeV. Thus a correction for energy response of the calorimeter can be calculated from fun-

Figure 3. Construction of a thin-foil calorimetric dosimeter.

damental theory and applied with reasonable certainty.

The design and construction of a practical calorimeter is shown in Figure 3. The circular beam intercepting foil and the heat leaks are cut from a single piece of copper foil. A copper-constantan thermocouple is used which produces an e.m.f. of 40 microvolts per degree centigrade. The cold junction of the thermocouple is made on a copper foil electrically insulated from, but in good thermal contact with the reference system for the heat leaks. This helps to isolate the cold junction from the external environment. The design shown has a time constant of about 3 sec and a sensitivity of $1/40^{\circ}\text{C}$ or one microvolt per 10^{-8} amperes of beam current. With modern amplifiers having noise levels of the order of one nanovolt, beam currents of 10^{-9} amperes may be measured and changes of the order of $2 \cdot 10^{-11}$ amperes may be resolved. The calorimeter may be designed for greater sensitivity but at the expense of a longer time constant and slower response.

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65111-8

PHARMACODYNAMICS OF SOME TECHNETIUM-99m PREPARATIONS*

By

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Technetium-99m assumes increasing importance as a diagnostic agent for organ visualization by scintiscanning as additional preparations, easily and quickly produced, become available. Publications from several medical centers¹⁻⁶ have shown the usefulness of ^{99m}Tc as NaTcO₄ in demonstrating brain lesions; and clinical trial has established the efficacy of ^{99m}Tc for visualizing the thyroid with pertechnetate,^{1,2,7,8} the liver, spleen, and bone marrow with a colloidal sulfur preparation,^{1,2,8,9-11} the kidneys with an iron complex, and the lungs and placenta with albumin preparations.^{8,12,13} In addition, the lungs have been visualized in a few patients using a gaseous form of technetium, and cisternography appears practicable by the intrathecal injection of pertechnetate.^{7,8} The present scope of organ visualization with ^{99m}Tc is illustrated in Figure 1.

Technetium-99m was initially selected for investigation solely on the basis of its favorable physical properties. Briefly summarized these physical properties consist of a 140 keV gamma radiation (0.92 photon/disintegration) with an absence of particle radiation; HVL in tissue = 4.6 cm, in lead = 0.3 mm, in NaI = 2.4 mm; six hour physical half-life; 67 hour shelf half-life as the daughter of molybdenum-99, from which it is easily eluted in the clinical laboratory. Thus, ^{99m}Tc is readily detectable, easily collimated and shielded, deposits little energy in tissue, is conveniently handled in the laboratory with little hazard of contamination and radiation exposure to personnel, and is continuously available.

Technetium-99m is eluted from the ⁹⁹Mo, ^{99m}Tc generator either as sodium pertechnetate or pertechnic acid depending on whether the eluting solution is sodium chloride or hydrochloric acid. When administered to laboratory animals as pertechnetate, the initial biological pattern is similar to that of iodide. This distribution pattern is made use of for brain and thyroid imaging.

Although the pertechnetate ion does not combine actively with other compounds, it is readily reduced to lower valence states that are more reactive. Such alterations of chemical form sometimes produce changes in the biological localization that may be useful for visualization of specific organs or areas. Chemical preparation of a radioisotope as short-lived as ^{99m}Tc must necessarily be limited to processes requiring no more than 24 hours for completion. Preference obviously is for procedures involving about 1 hour or less in the interests of conserving radioactivity, reducing radiation exposure to the radiopharmacist, and minimizing patient scheduling problems.

* This report is taken from a paper that was presented before the Ninth Oak Ridge Institute of Nuclear Studies Symposium in Medicine - Radioactive Pharmaceuticals, November 1-5, 1965.

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Figure 1. A. Thyroid gamma scintillation camera study (scintiphotograph) using 1 millicurie of pertechnetate- 99m and an exposure of 5 minutes with a single pinhole collimator. A cold nodule is demonstrated in the right lower lobe, the left lobe is diminutive because of previous surgery. B. Lateral brain scan with 10 millicuries of pertechnetate- 99m . The scanning time was 15 minutes on the Argonne Cancer Research Hospital's brain scanner. A large meningioma is evident in the parietal region. C. Normal posterior cisternal scan with 2 millicuries of intrathecal pertechnetate- 99m injected directly into the cistern in a patient pretreated with perchlorate. The scan was made 4 hours after injection. D. Inhalation scintiphotograph using vaporized technetium- 99m . This anterior view of the chest was made with a 1 minute exposure within 10 minutes after inhalation of 350 microcuries. E. Posterior lung scan with ^{99m}Tc macro-aggregated-albumin. Decreased uptake is evident in the right lower lobe. F. Placenta scintiphotograph using 1 millicurie of ^{99m}Tc -albumin. This 2 to 3 minute exposure was made with the 3 inch 1059 hole multi-aperture collimator. The placenta is located in the left upper quadrant of the uterus. Activity is visible in the bladder. G. Ten second scintiphotograph of the heart blood pool showing the superior vena cava, right auricle, right ventricle, and main pulmonary artery. H. Anterior multiple organ scintiphotography. This 1 minute exposure was made with Tc-sulfur colloid in the liver, Tc-macro-aggregated-albumin in the lung, and unaggregated albumin in the heart blood. The clear space around the heart is a pericardial effusion. I. Anterior scintiphotograph of the liver with 2 millicuries of ^{99m}Tc -sulfur colloid. This 1 to 2 minute exposure shows a metastasis in the lateral inferior aspect of the right lobe. J. Scintiphotograph of a normal spleen and left lobe of liver using 2 millicuries of ^{99m}Tc -sulfur colloid. The exposure time was 2 minutes. K. Normal bone marrow uptake of ^{99m}Tc -sulfur colloid in the pelvis of a 6-year-old child. A 5 minute exposure was used with a dose of 500 microcuries. L. Posterior oblique view of the left kidney in a patient with polycystic disease. This 4 minute exposure was made 1 hour after intravenous injection of 1 millicurie of ^{99m}Tc iron complex.

Clinical use of short-lived isotopes for intravenous administration creates problems in pharmacy not solvable by conventional pharmaceutical methods. At this institution, where no patient reactions attributable to the injected solution have been observed in approximately 1000 administrations, the following protocol is observed. All short-lived radioisotopes intended for intravenous use in humans are prepared by a registered pharmacist who also has some training in the handling and assay of radioisotopes. Care is taken to use sterile, pyrogen-free reagents during the preparation, and to further sterilize the product by filtration through a sterile 200 m μ millipore filter. From time to time, sterility and pyrogen testing are done on reagents. For intrathecal use pertechnetate is further sterilized by autoclaving.

PERTECHNETATE

After intravenous injection as pertechnetate, ^{99m}Tc is rapidly localized in the stomach, urinary bladder, thyroid and salivary glands, as illustrated by Figure 2. Similar patterns are observed after intraperitoneal, oral, or intramuscular administration although localization is slower. In man, disappearance from the plasma into the extravascular spaces proceeds fairly

Figure 2. Serial scans of a mouse after intravenous injection of pertechnetate- ^{99m}Tc , showing the rapid concentration of ^{99m}Tc in the thyroid and salivary glands, stomach, and urinary bladder. Scans made at 10 minutes (left), 1, 6, and 24 hours.

rapidly for rather more than 50 per cent of intravenously injected pertechnetate, the half-time being about 15 minutes. The remainder of the radioactivity stays in the plasma with a half-time of approximately 3 hours. After oral administration in the same subject, the level of ^{99m}Tc in the blood reaches its highest value in approximately 30 minutes, and is about one-half the radioactivity present at the same, and subsequent times after intravenous injection. The rate of absorption and resultant blood level of ^{99m}Tc will vary after ingestion with the presence or absence of food in the stomach. In the same individual, very rapid colonic absorption was indicated: the blood level was at a maximum when the first sample was withdrawn 5 minutes after introduction of the pertechnetate into the large bowel through a colostomy. The blood disappearance curves in these three situations are shown in Figure 3. Intestinal absorption for pertechnetate is similarly indicated by the study illustrated in Figure 4 where location

Figure 3. Disappearance of ^{99m}Tc from the plasma of one subject after three routes of administration for pertechnetate- ^{99m}Tc .

Figure 4. Serial scans of a mouse after oral administration by stomach tube of pertechnetate- ^{99m}Tc , showing intestinal absorption followed by localization in the stomach, urinary bladder, thyroid and salivary glands. Intestinal excretion is prominent in the 24 hour scan. Scans at 8 minutes (left), 1, 5, and 24 hours.

of ^{99m}Tc in a mouse was followed by scanning at intervals after oral administration.

In the mouse, the highest concentration of ^{99m}Tc during the first several hours¹⁴ after intravenous injection as pertechnetate is in the thyroid. Thereafter, radioactivity in the gland decreases in proportion to the serum level. Representative values are: 30 min: 115 per cent/g; 1 hour: 480 per cent/g; 2 hours: 120 per cent/g; 3 hours: 90 per cent/g. Other tissue concentrations are given in Table 1. No evidence for organification of technetium by the thyroid gland has been found on electrophoresis or chromatography of tissue homogenates. In plasma and liver homogenates, part of the ^{99m}Tc is associated with protein fractions.

Table 1
TECHNETIUM- 99m IN MOUSE TISSUES AFTER INTRAVENOUS
INJECTION AS PERTECHNETATE

Tissue	^{99m}Tc per gram tissue, %			
	0.5 hour	1 hour	2.5 hours	24 hours
Blood	$8.61^* \pm 0.60^\dagger$	5.35 ± 0.60	5.04 ± 0.41	0.19 ± 0.14
Brain	0.45 ± 0.05	0.19 ± 0.02	0.21 ± 0.03	0.03 ± 0.01
Heart	3.10 ± 0.28	2.37 ± 0.45	1.37 ± 0.11	0.16 ± 0.03
Intestine	3.16 ± 0.20	4.22 ± 0.30	3.78 ± 0.46	1.45 ± 0.79
Kidney	4.39 ± 0.14	2.6 ± 0.25	2.58 ± 0.07	1.15 ± 0.11
Liver	7.12 ± 0.33	3.8 ± 0.50	4.25 ± 0.47	1.03 ± 0.05
Lungs	7.25 ± 0.34	4.2 ± 0.60	3.57 ± 0.41	0.52 ± 0.06
Muscle	1.84 ± 0.08	1.8 ± 0.60	1.01 ± 0.09	0.23 ± 0.13
Ovary	2.24 ± 0.25	2.7 ± 0.30	1.76 ± 0.39	0.66 ± 0.10
Spleen	3.99 ± 0.30	2.2 ± 0.25	2.07 ± 0.24	0.14 ± 0.03
Stomach and contents	57.20 ± 3.62	106.7 ± 37	66.82 ± 8.87	5.60 ± 1.33

* Average of 5 animals for each determination.

† Standard deviation of the mean.

In human subjects, excretion of ^{99m}Tc during the 24 hours after administration as pertechnetate occurs largely in the urine and amounts to about 25 per cent.⁷ Fecal excretion during the next 48 hours may total as much as 25 to 35 per cent. Approximately 50 per cent is eliminated at 50 hours, and the remainder is excreted at a somewhat slower rate with a half-time of 75 hours. Using ^{95m}Tc ($t_{1/2} = 60$ days), Nelp¹⁵ has determined that complete excretion, which cannot be determined with the short-lived ^{99m}Tc , takes place within less than 2 weeks. This consideration is of consequence only in assessing long-term radiation effects from the possible accumulation of ^{99}Tc with repeated doses of ^{99m}Tc . This is obviously no cause for concern when pertechnetate is used: the approximate radiation dosage from 1 mc intravenous pertechnetate- 99m is 10 mrad to whole body, 300 mrad to stomach, and 100 mrad to thyroid.¹⁶

Effectiveness of pertechnetate- 99m for brain scanning depends on its distribution in the extracellular spaces and exclusion from normal brain tissue. Compared with other radiopharmaceuticals, pertechnetate- 99m gives greater visualization of normal anatomic structures

(Figure 1-B), a feature that is disturbing to some interpreters but gratifying to those others who use it for orientation and the additional functional information it may provide.¹⁷ The actual technique of brain scanning varies considerably and individual preferences have been reported by several groups.³⁻⁶

Although less widely used at present for clinical studies of the thyroid than for brain scanning, pertechnetate-99m may prove to have even more interesting possibilities in this area. Good images of the thyroid gland are obtained from 1 mc ^{99m}Tc in euthyroid and hyperthyroid states, cold nodules (Figure 1-A), and hyperactive multinodular goiters, with the advantage over ¹³¹I that the gland receives about 1/1000 the radiation dosage.⁷ Since concentration of pertechnetate-99m in the thyroid appears to reflect the activity of the ion-concentrating mechanism, evaluations of the uptake repeated at intervals have proved useful in the medical management of hyperthyroidism for determining the effectiveness of treatment with blocking agents.⁷ Another potentially useful application may be separation of the magnitude of the organification process from that of ion-concentration by employing pertechnetate-99m and radioiodine as a double label while measuring their uptake versus time.

The two following examples suggest some other possible applications that undoubtedly will continue to be found for ^{99m}Tc as the stable, easily obtained pertechnetate. Cisternography or myelography, illustrated by Figure 1-C, is possible after intrathecal injection. Absorption from the subarachnoid space is greatly reduced by pretreatment with perchlorate.^{7,8} Cardiac filling and emptying has been observed after intravenous pertechnetate using the motion picture and Anger camera in combination.¹⁸

Although pertechnetate-99m may be used directly for intravenous injection by elution from the generator with isotonic saline after first sterilizing by filtration through a millipore membrane, we continue to prefer extraction into methyl-ethyl-ketone. This process not only results in complete control of solution volume, but provides ^{99m}Tc in a more convenient form for processing into other chemical states, and also eliminates the eluted ⁹⁹Mo. This latter, while not interfering with the scanning, adds to the patient's radiation dose, which should always be minimized by any practical means. Eluted ⁹⁹Mo, which seems to be less from generators manufactured with ⁹⁹Mo separated from fission products than from that produced by neutron irradiation of molybdenum, remains in the aqueous phase when the eluate is treated in the following manner:

Preparation of Stock Solution

1. Pour 25 ml of 0.1 N HCl or isotonic saline on the column. Collect eluate in a separatory funnel fitted with a side arm.
2. Add to funnel through side arm 12.5 ml of 15N NaOH solution. Mix with magnetic stirrer.
3. Add through side arm 25 ml of methyl-ethyl-ketone (redistilled weekly) and mix for 3 minutes.
4. Allow to stand until ketone and aqueous layers separate completely.
5. Collect the ketone layer containing ^{99m}Tc and determine the activity per ml.

This is the stock solution (A). Adherent base may be removed from the ketone solution by decanting or passing through dry alumina.

Preparation of Pertechnetate-99m for Intravenous Injection

1. Take required aliquot of stock solution (A) in a sterile beaker and evaporate to dryness in a stream of filtered air by gentle heating on a hot plate.

2. Add 3 cc (or any desired volume) of sterile pyrogen-free isotonic saline to beaker and shake briefly.
3. Take up the solution in a syringe and filter into a second sterile syringe through a sterile 0.2 μ millipore filter using microfiber glass prefilter. Measure the radioactivity.

TECHNETIUM-99m-SULFUR COLLOID

Intravenous ^{99m}Tc -sulfur colloid rapidly removed by the reticuloendothelial system, may be utilized for visualization of the liver, spleen, and bone marrow (Fig. 1-I, J, K). Tissue distribution studies in the mouse (Table 2) suggest that there is little translocation or excretion from the original sites of deposition. Since little absorption occurs when other routes of administration are employed (Figure 5), it is necessary to take care in making the intravenous injection.

Figure 5. Localization of Tc-sulfur colloid at 1 hour after administration by various routes. A (left). Liver and spleen after intravenous injection showing some urinary excretion. B. Intradermal injection into left forearm possibly showing some uptake by the lymphatics. C. Intraperitoneal injection. D. Intramuscular injection. E. Oral (stomach tube) administration indicating no intestinal absorption.

tions. Data from total-body counting and excreta recovery in the human (Figure 6) are indicative of long-term retention of this preparation. Urinary-fecal partition for the same subject is shown in Table 3. Based on 85 per cent permanent retention in the liver, the estimated radiation dosage is approximately 400 mrad/mc. One to 3 mc quantities for a liver scan consequently result in minimal radiation, and even the 10 to 15 mc's preferred dose for bone marrow visualization¹¹ is very tolerable. Disappearance of ^{99m}Tc in this form from blood and plasma is rapid during the first minutes after intravenous injection. Plasma concentrations at 1 hour for the 5 subjects shown on the normalized curve in Figure 7 range between 0.0019 per cent/ml and 0.0159 per cent/ml and average 0.0057 per cent/ml. A transient association of ^{99m}Tc with the cells is suggested by an increase in the plasma/whole blood ratio to about twice the initial value within 1 hour. A large fraction of the ^{99m}Tc in the cellular fraction may be contained in the buffy coat. In one patient with extensive liver destruction, this component held 80 per cent of the blood

Table 2
DISTRIBUTION OF ^{99m}Tc IN THE MOUSE AFTER INTRAVENOUS ADMINISTRATION OF Tc-SULFUR COLLOID

Tissue	% of dose per organ			% of dose per gram		
	1 hour	24 hours	48 hours	1 hour	24 hours	48 hours
Blood				$2.59 \pm 0.46^*$	$0.76 \pm 0.06^\dagger$	$0.18 \pm 0.015^\ddagger$
Brain	0.02 ± 0.008	0.015 ± 0.002	0.01 ± 0.0	0.06 ± 0.013	0.04 ± 0.007	0.02 ± 0.005
Heart	0.09 ± 0.02	0.08 ± 0.02	0.04 ± 0.005	0.84 ± 0.026	0.76 ± 0.20	0.35 ± 0.077
Intestines	1.49 ± 0.29	5.90 ± 1.26	1.44 ± 0.07	0.57 ± 0.018	3.09 ± 0.84	0.72 ± 0.14
Kidney	0.60 ± 0.12	1.06 ± 0.06	1.32 ± 0.05	2.33 ± 0.15	4.06 ± 0.06	5.45 ± 0.14
Liver	70.52 ± 1.96	73.74 ± 2.37	84.68 ± 0.74	60.49 ± 4.85	81.65 ± 5.44	72.77 ± 3.75
Lungs	0.51 ± 0.10	0.23 ± 0.04	0.14 ± 0.01	2.93 ± 1.41	1.37 ± 0.21	0.83 ± 0.30
Muscle				0.25 ± 0.034	0.24 ± 0.04	0.16 ± 0.005
Ovary	0.03 ± 0.018	0.02 ± 0.003	0.02 ± 0.025	0.47 ± 0.14	0.60 ± 0.10	0.33 ± 0.10
Skin				0.38 ± 0.075	0.24 ± 0.03	0.19 ± 0.05
Spleen	1.67 ± 0.25	0.98 ± 0.12	1.87 ± 0.15	19.07 ± 2.0	18.94 ± 2.28	35.68 ± 1.65
Sternum	0.34 ± 0.22	0.13 ± 0.03	0.23 ± 0.07	2.46 ± 0.40	1.67 ± 0.27	2.63 ± 1.14
Stomach	2.13 ± 0.12	1.42 ± 0.33	0.30 ± 0.07	6.25 ± 0.42	3.11 ± 0.61	0.86 ± 0.20
Carcass	13.47 ± 1.22	10.52 ± 1.90	5.42 ± 0.30			
Excreta	0.67 ± 0.47	8.30 ± 1.55	4.41 ± 0.67			

* Values at 1 hour are the average for 7 mice $\pm$ standard error.

† Values at 24 hours are the average for 6 mice $\pm$ standard error.

‡ Values at 48 hours are the average for 5 mice $\pm$ standard error.

Figure 6. Total body retention of ^{99m}Tc after intravenous injection of 4 mc ^{99m}Tc -sulfur colloid. ● = total body counts. x = injected dose minus recovered excreta.

Table 3
EXCRETION OF ^{99m}Tc AFTER INTRAVENOUS ADMINISTRATION
OF Tc-SULFUR COLLOID

Time in hours	% of injected ^{99m}Tc		
	Urine	Feces	Cumulative total
0-27	0.67		0.67
27-50	0.97	1.08	2.72
50-75	1.23	1.73	5.68
75-92	1.53	0.38	7.59

radioactivity 10 minutes after injection. Saliva/plasma ^{99m}Tc ratios remain considerably less than 1:1 (around 0.5) for the sulfur colloid. The chemical nature of this preparation is difficult to identify because of inherent problems of isolation when dealing with carrier-free amounts of any short-lived radioisotope. Using milligram quantities of ^{99}Tc in 2N to 4N hydrochloric acid, the heptasulfide, Tc_2S_7 , is precipitated.¹⁹ Tracer quantities of short-lived technetium isotopes are also coprecipitated from acid solution with the sulfides of many elements.²⁰ It thus appears probable that in this radiopharmaceutical ^{99m}Tc exists as the insoluble Tc_2S_7 carried on colloidal sulfur and stabilized with gelatin. When treated with base the sulfide decomposes to pertechnetate. For this reason, complete neutralization of the preparation before injection is not desirable and should be carried out so that pH 6 is never exceeded and no back titration is necessary. Varying amounts of pertechnetate are formed if pH adjustment is not made carefully; this causes a circulating background and stomach shadow resulting in poor scan quality. While difficult to measure accurately, actual particle-size in this preparation must have an upper limit of less than 50 μ because losses of radioactivity with this size of millipore filter are no greater than losses with larger pore filters. The marked retention of ^{99m}Tc by a 10 μ

Figure 7. Disappearance of ^{99m}Tc from plasma after intravenous injection of Tc-sulfur colloid. The 1 hour value, which has been set equal to 1, ranged between 0.0019%/ml and 0.0159%/ml and averaged 0.0057%/ml for the 5 subjects presented.

filter may be due to physical processes occurring on the membrane rather than to the holding back of larger particles. Attempts to measure sulfur indicate the presence of only a few micrograms in the final preparation, many times less than was once used without ill effect for the treatment of arthritis.²¹ In mice, where the volume per kilogram administered is about one hundred times that used on patients, no acute harmful results have been encountered from the preparation: Animals appeared healthy after as many as 10 such injections over a period of several weeks.

Preparation of the ^{99m}Tc -sulfur colloid used for intravenous injection at Argonne Cancer Research Hospital

1. Remove aliquot of stock solution containing desired radioactivity to a 25-ml Erlenmeyer flask; evaporate to dryness by mild heating in a stream of filtered air.
2. Add 2 ml of 1 per cent solution of gelatin* in 1N HCl, prepared immediately before use.
3. Heat gently to dissolve gelatin. Add 40 ml H_2S gas measured in syringe

* Vee Gee Pharmaceutical gelatin; Bloom value = 225; Viscosity of 2 per cent gel = 65; Lime Processed Hide gelatin.

fitted with 3-way needle adapter inserted into flask stopper and allow to stand at room temperature for 30 minutes.

4. Adjust pH with 1N NaOH to pH 5.5 - 6.0. (This adjustment should be made carefully so that pH never exceeds 6 which could result in formation of pertechnetate ion.)
5. Take the neutralized solution in syringe and pass through sterile 0.22 μ filter using microfiber glass prefilter type AP20, 22 mm.
6. Collect the filtrate directly in another sterile syringe and measure the activity. Yield should be between 90 and 95 per cent. The solution volume is 4 to 5 ml.

TECHNETIUM-99m-IRON-59 COMPLEX

When technetium-99m in 2.5N hydrochloric acid is reduced from the $+7$ valency of pertechnetate by treatment with ascorbic acid, it combines with iron to form a substance most of which is promptly excreted in the urine after intravenous injection. Some ^{99m}Tc remains fixed in the kidney and may be utilized to produce scans of the type shown in Figure 1-L, 8. The former scan, showing a large cyst in the lower pole of the right kidney, was made 24 hours after the patient

Figure 8. Renal scintiphotography with the Tc-Fe complex. A. Posterior view of the kidneys 24 hours after intravenous injection of 4 millicuries Tc. Fifty thousand counts were collected with an exposure of 4 minutes using the gamma scintillation camera with low energy multiaperture collimator. A mass is noted in the lateral lower pole of the right kidney; the left kidney is normal. The white circular outline is the field of view. B. Oblique view with the right posterior flank toward the camera crystal. The lesion is even more evident, and causes the truncated appearance of the lower pole. The intravenous pyelogram in this case showed minimal calyceal deformity, and was interpreted by some radiologists as normal. A subsequent selective right renal arteriogram demonstrated a renal cyst.

received 4 mc of ^{99m}Tc . An estimate that 19 per cent of the injected dose is retained in the kidneys was made in a second patient by comparison with ^{99m}Tc in a kidney phantom. Total body counting at this same time indicated 38 per cent of injected dose retained. No significant radio-

activity was noted in the liver or intestine at the time of kidney scanning. Thus about half of the retained ^{99m}Tc appears to be localized in the kidneys in this case. Total body retention by this subject was determined from daily counts made in the Argonne Cancer Research Hospital's total body counting facility and also by quantitative excreta recovery. The total body retention curve is shown in Figure 9. Based on these data, the estimated radiation to the kidney is less

Figure 9. Total body retention of ^{99m}Tc after intravenous injection of 4 mc Tc-Fe complex to adult male patient. ● = total body counts. x = injected radioactivity minus recovered excreta.

than 500 mrad/mc. Analysis of mouse tissues after intravenous injection of the Tc-Fe preparation show the highest concentration of ^{99m}Tc per gram of tissue in the kidney and some concentration in the liver and intestine (Table 4). Similar experiments with a double label of ^{99m}Tc and ^{59}Fe reveal a greater excretion of ^{99m}Tc and 30-fold concentration of ^{59}Fe (60 per cent of injected dose) over ^{99m}Tc in the liver, although the two isotopes are approximately equal in all other tissues examined (Table 5). A radioautograph from ^{99m}Tc in a kidney removed 45 minutes after intravenous administration of the Tc-Fe complex shows localization of the radioactivity in the cortex (Figure 10).

Initial disappearance of ^{99m}Tc from the plasma is very rapid for the Tc-Fe complex, 50 per cent or more having usually left the blood by the time of the first sampling at 3 to 5 minutes. The average value at 1 hour was 0.0059 per cent injected dose/ml (range 0.0039-0.0076) for the

Table 4
^{99m}Tc DISTRIBUTION IN MOUSE TISSUES AFTER INTRAVENOUS
 INJECTION OF Tc-Fe COMPLEX

Organ	% of injected dose per organ		
	1 hour	24 hours	48 hours
Heart	0.05 [*] 0.04 - 0.06 [†]	0.01	0.01
Lung	0.18 0.15 - 0.20	0.04 0.03 - 0.05	0.03 0.02 - 0.03
Liver	2.18 2.08 - 2.22	0.66 0.51 - 0.73	0.40 0.44 - 0.55
Spleen	0.09 0.08 - 0.09	0.03 0.02 - 0.04	0.02
Stomach	0.62 0.56 - 0.71	0.10 0.06 - 0.13	0.03 0.02 - 0.04
Intestines	4.18 3.64 - 4.52	0.45 0.43 - 0.50	0.18 0.13 - 0.23
Kidneys	3.15 2.11 - 4.35	0.54 0.36 - 0.64	0.28 0.23 - 0.32
Brain	0.03 0.02 - 0.04	0.02 0.01 - 0.02	0.01
Sternum	0.07 0.05 - 0.09	0.02	0.01
Carcass	19.36 12.96 - 28.91	1.42 1.31 - 1.59	0.91 0.81 - 0.97

* Average for 4 mice.

† Range.

8 subjects shown on the normalized plasma disappearance curve in Figure 11.

Reaction of technetium with iron in acid solution occurs on the addition of a reducing agent. Without such an agent, ^{99m}Tc is virtually quantitatively removed by an anion exchange resin. In the presence of a reducing agent, 50 to 60 per cent of the ^{99m}Tc remains unattached to the resin. This may be related to the observation by other investigators^{20,22} that reduced Tc is carried on ferric ion, although the iron in our preparation does not react with potassium ferrocyanide to give the blue color indicative of ferric iron. The white precipitate formed between this reagent and ferrous ion is obtained before neutralization. After neutralization, neither color forms on addition of potassium ferrocyanide nor does the solution have the characteristic brownish color of ferric iron. When highly concentrated, its appearance is more nearly that of an iron sol. If the preparation is made with ⁵⁹FeCl₃, less than 10 per cent ⁵⁹Fe is retained by the anion exchange column used to remove pertechnetate. On electrophoresis and subsequent radioautography of the preparation the two isotopes seem to remain associated and to have a mobility different from either sodium pertechnetate or ferric chloride.

Table 5

RELATIVE LOCALIZATION OF RADIOISOTOPES
IN MOUSE TISSUES AFTER INTRAVENOUS
INJECTION OF ^{99m}Tc - ^{59}Fe COMPLEX

Tissue	Ratio of ^{99m}Tc : ^{59}Fe	
	Mouse No. 1	Mouse No. 2
Blood	0.63	0.50
Brain	1.31	0.53
Heart	0.34	0.20
Intestines	0.71	1.03
Kidney	0.83	0.57
Liver	0.036	0.026
Lungs	0.46	0.62
Muscle	1.24	0.59
Ovary and fat	2.01	1.60
Skin	1.86	0.62
Spleen	0.36	0.53
Stomach	0.96	0.76
Excreta	5.32	8.96

Preparation of ^{99m}Tc -Fe complex

1. Evaporate the desired quantity of ^{99m}Tc contained in methyl-ethyl ketone to dryness in a small flask using gentle heat and a stream of air.
2. To the flask add 2 ml 2.5N HCl, 0.1 ml per cent ascorbic acid solution, and 0.1 ml of a 1 per cent solution of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 1 per cent HCl. Set aside at room temperature for 30 minutes.
3. Neutralize with NaOH to pH 7.2-7.8 and pour through a small IR-4B resin column in the chloride phase.
4. Filter eluate through a sterile 0.22 μ millipore membrane into a sterile syringe.

OTHER TECHNETIUM-99m PREPARATIONS

Methods of preparation and administration are largely completed for three other preparations of ^{99m}Tc that may prove to be useful radiopharmaceuticals. These are a gaseous form for inhalation studies, Tc-labeled albumin, and technetium thiocyanate. Clinical use of these compounds has been shown to be practical, and localization and retention studies in animals and man are in progress.

Preparation of gaseous technetium-99m

In preparing technetium-99m in gaseous or vapor form for possible use in lung function studies, pertechnetate in methyl-ethyl-ketone is dried onto a platinum (not tungsten) filament, and then vaporized by heating the filament to a bright yellow color by passage of an electric

Figure 10. Radioautograph from ^{99m}Tc in rabbit kidney (5 mm slice) showing localization of radioactivity in cortex 45 minutes after intravenous injection of Tc-Fe complex.

current. At this temperature ~ 90 per cent of the radioactivity leaves the filament. This behavior suggests that NaTcO_4 either may be sublimed without decomposition, as occurs with KTcO_4 at around 1000°C ,²³ or is first decomposed to TcO_2 which distills rapidly²⁴ at 1100°C . Observations in animals showed that a substantial fraction of the inhaled ^{99m}Tc remained in the lungs 30 minutes after inhalation. Clinical experiments confirm this observation. Inhaled ^{99m}Tc remains in the lungs, disappearing gradually over the next hour, with localization in the stomach and salivary glands. This would be consistent with either a slow absorption of pertechnetate or a gradual conversion of the oxide to pertechnetate.

Presumably the material follows the air flow into the lungs, condensing on the walls of the

Figure 11. Disappearance of ^{99m}Tc from the plasma after intravenous injection of Tc-Fe complex. Values at 1 hour for these 8 patients ranged between 0.0036 - 0.0076 per cent injected dose/ml (ave: = 0.0059%).

air passages as the radioactive molecules diffuse outward from the air stream. Whether a preparation with these characteristics is generally suitable for pulmonary function studies is not entirely clear at the moment, but in a normal individual inhaling rapidly, uniform tagging of the lung field takes place (Figure 1-D).

Pertechnetate in either basic or acidic solution does not combine with albumin more than 1 or 2 per cent. Use of a reducing agent in acid medium produces a rapid addition of ^{99m}Tc to albumin. Because Tc in lower valence states has an affinity for other substances, it is most desirable to limit the reaction medium to those chemicals actually required in the reaction; namely, Tc, albumin, and the reducing agent. This differs somewhat from the procedure of Stern,²⁵ which requires the presence of iron for tagging. For perfusion scanning, specific activity of the preparation should be about 10 mc/mg of albumin, and the Tc must remain firmly bound when heated to produce macro-aggregated particles. These requirements are met satisfactorily by using the semi-micro method described below. Limited clinical experience shows that this is a useful and safe material (Figure 1-E, F, G, H).

Preparation of Tc-labeled albumin

1. Add the required amount of ^{99m}Tc in methyl-ethyl-ketone to a small test tube (total volume 4 ml) and evaporate to dryness with gentle heating in a stream of air.
2. To the tube add:
1 ml 2.5N HCl
0.5 ml 5 per cent ascorbic acid solution
Allow to stand at room temperature 30 minutes or longer to reduce the pertechnetate.
3. To the above solution add with mixing (using a magnetic stirrer) 0.1 ml of 1 per cent albumin solution. The albumin is a sterile solution prepared by diluting sterile normal human serum albumin (12.5 g/50 ml) with sterile saline (1 ml diluted to 25 ml).
4. After mixing for 15 minutes add 0.2 ml of 10N NaOH to the albumin solution to bring to about pH 1.
5. Slowly add 1N NaOH until pH 5.5 is reached. This is accomplished by inserting into the solution a Sargent No. S-30070-10 combination electrode to which is fastened a long glass capillary tube for addition of the base. The final volume will be approximately 2 ml.
6. When the preparation is to be used for blood pool (placenta) visualization, 1N NaOH is added until pH 6 is reached. The solution is then passed through a small anion exchange column (Amberlite IR-4B in chloride phase) to remove pertechnetate, and sterilized by filtering through a sterile 0.22 μ millipore membrane.
7. When the preparation from step 5 is to be aggregated, it is sterilized by passage through a sterile 0.22 μ millipore membrane and collected in a sterile vial. Macro-aggregation of the albumin takes place on heating the vial in boiling water for 4 minutes. The aggregated albumin is prepared for injection by passage through a small sterile IR-4B column to remove any pertechnetate. The column is rinsed with 1 ml sterile saline which is collected with the albumin in a sterile vial or syringe.

Preparation of Technetium thiocyanate

Technetium thiocyanate was prepared by the analytical method of Howard and Weber,²⁶ with the modifications described. Localization of this material in the mouse varied with individual preparations until it was appreciated that thiocyanates are soluble in fat solvents and particular care must be taken to avoid contamination with such substances (e.g., stopcock grease). A preparation with predictable behavior²⁷ was obtained by dissolving the thiocyanate in a commercially available fat emulsion designed for intravenous use. This localized regularly and reproducibly in the mouse liver, and tissue distribution studies are shown in Table 6.

In the clinical situation localization is much slower (Figure 12), the optimal time for liver scanning being 4 to 6 hours after injection. In a patient with severe liver necrosis no uptake was found at 4 hours,²⁸ and in a patient with hepatoma there was substantial localization in the tumor.^{1,2} Localization of this material thus appears to be a function of the polygonal cell rather than of the Kupfer cell. In one patient with a biliary fistula, 20.5 per cent of the injected ^{99m}Tc

Table 6

DISTRIBUTION OF ^{99m}Tc IN MOUSE TISSUES AFTER INTRAVENOUS ADMINISTRATION OF $\text{Tc}(\text{SCN})_5$ IN LIPOMUL[®]

	% of injected dose per gram of tissue							
	0.5 hour [*]	1 hour [*]	2.5 hours [*]	5 hours [†]	8 hours [‡]	10 hours [‡]	15 hours [*]	24 hours [†]
Brain	0.27 ± 0.10 [§]	0.32 ± 0.05	0.14 ± 0.01	0.11 ± 0.01	0.11 ± 0.02	0.06 ± 0.01	0.07 ± 0.01	0.03 ± 0.04
Blood	7.72 ± 0.74	8.12 ± 0.70	3.66 ± 0.25	4.44 ± 0.45	3.30 ± 0.15	2.13 ± 0.28	2.10 ± 0.16	0.72 ± 0.18
Heart	4.69 ± 0.31	5.42 ± 0.80	2.18 ± 0.08	1.48 ± 0.11	1.16 ± 0.07	0.79 ± 0.01	0.94 ± 0.10	0.48 ± 0.07
Intestine and contents	2.04 ± 0.16	1.95 ± 0.04	3.34 ± 0.20	4.36 ± 0.92	8.39 ± 0.62	3.07 ± 0.27	2.79 ± 0.07	2.99 ± 0.09
Kidneys	10.06 ± 0.58	7.28 ± 0.56	6.76 ± 0.39	7.00 ± 0.79	5.75 ± 0.22	5.74 ± 0.09	5.63 ± 0.13	3.52 ± 0.40
Liver	44.77 ± 0.73	39.73 ± 2.43	41.04 ± 3.37	34.33 ± 1.59	14.60 ± 0.98	18.22 ± 0.73	15.98 ± 0.53	15.17 ± 0.85
Lungs	5.34 ± 0.25	4.52 ± 0.25	2.84 ± 0.15	2.21 ± 0.07	2.58 ± 0.23	1.25 ± 0.07	1.03 ± 0.20	0.69 ± 0.04
Muscle	0.66 [¶]	0.95 [¶]	0.84 [¶]	0.67 ± 0.28	0.61 ± 0.05	0.37 ± 0.04	0.50 ± 0.05	0.15 ± 0.11
Ovary and fat	1.75 ± 0.52	2.31 ± 0.49	1.70 ± 0.23	1.16 ± 0.63	1.10 ± 0.19	0.45 ± 0.08	0.51 ± 0.05	0.27 ± 0.17
Spleen	4.84 ± 0.38	3.77 ± 0.19	2.53 ± 0.14	2.21 ± 0.31	3.12 ± 0.26	0.97 ± 0.03	1.28 ± 0.13	0.73 ± 0.06
Stomach	20.58 ± 2.89	22.67 ± 5.47	15.40 ± 1.94	9.45 ± 0.91	12.19 ± 1.10	6.04 ± 1.44	3.03 ± 0.57	1.81 ± 0.04
Total recovery in % of injected dose								
Carcass	33.58 ± 2.10	29.30 ± 1.97	23.83 ± 0.80	15.10 ± 1.19	19.42 ± 0.85	10.69 ± 0.60	10.50 ± 0.58	7.23 ± 0.54
Excreta	0.61 ± 0.10	0.76 ± 0.98	2.02 ± 0.65	9.26 ± 2.26	18.98 ± 2.20	51.23 ± 2.20	58.20 ± 2.85	65.88 ± 0.95

^{*} 4 mice.[†] 3 mice.[‡] 5 mice.[§] Mean ± standard error.[¶] Single determination.

Figure 12. Relative count rates over liver and precordium following intravenous injection of $\text{Tc}(\text{SCN})_5$ in fat emulsion.

was recovered in the urine, 6.1 per cent in the feces and 14 per cent from the fistula by the end of 96 hours. The excretion of $^{99\text{m}}\text{Tc}$ after its intravenous administration as $^{99\text{m}}\text{Tc}(\text{SCN})_5$ by a group of eight hospitalized subjects is summarized in Table 7.

Table 7
EXCRETION OF $^{99\text{m}}\text{Tc}$ AFTER INTRAVENOUS ADMINISTRATION
OF $\text{Tc}(\text{SCN})_5$ DISSOLVED IN LIPOMUL*

Hours post-injection	% of injected $^{99\text{m}}\text{Tc}$		
	Urine	Feces	Cumulative total
0 - 24	16.2 [†] 7.4 - 24.1 [‡]	2.8 0.5 - 6.9	19.0 12.4 - 27.5
24 - 48	5.2 2.8 - 15.9	5.6 1.6 - 9.8	29.7 19.5 - 41.1
48 - 72	2.4 1.1 - 3.3	5.1 1.5 - 12.5	37.2 25.2 - 56.1

* Lipomul® I.V. — (cottonseed oil emulsion) — The Upjohn Company, Kalamazoo, Michigan.

[†] Average for 8 patients hospitalized for a variety of diseases.

[‡] Range.

Preparation of the thiocyanate dissolved in Lipomul® I.V. for intravenous use

1. Transfer the desired quantity of pertechnetate- $^{99\text{m}}$ in 0.1N HCl or isotonic saline to a separatory funnel that is fitted with a Teflon stop-cock and is of a shape that permits thorough mixing with a magnetic stirrer.

2. Adjust the volume and acidity to 25 ml with 2.5N HCl.
3. While mixing add in order:
 0.5 ml 1 per cent $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 1 per cent HCl
 2.5 ml 6.5 M ammonium thiocyanate
 0.5 ml 5 per cent ascorbic acid
 Continue stirring until the solution becomes colorless or only faintly pink.
4. Extract the thiocyanate into 15 ml n-butyl acetate and remove the aqueous layer.
5. Wash the butyl acetate twice with 10 ml portions of 2.2 M sodium dihydrogen phosphate monohydrate containing 0.5 ml of the ammonium thiocyanate solution in order to remove $\text{Fe}(\text{SCN})_2$. (The iron is used to prevent the formation of valencies lower than 5+ for technetium²⁶ and also serves as a useful color indicator for degree of reduction.)
6. Collect the butyl acetate solution in a sterile vial or beaker and remove the acetate by gentle evaporation at a low heat on a hot plate in a stream of filtered air.
7. Add 1 ml Lipomul® I.V. to the dry $^{99\text{m}}\text{Tc}(\text{SCN})_5$. Warm and shake vigorously for several minutes, then withdraw the solution into a sterile syringe.
8. Discharge this solution onto a small (1 x 0.3 cm) sterile anion exchange column (Amberlite IR-4B, chloride phase) and wash with 2 ml sterile saline. Use of the resin is necessary to remove pertechnetate produced on evaporation of the thiocyanate to dryness. The final solution has 25 per cent to 50 per cent of the initial radioactivity.

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INFLAMMATORY EFFECTS OF PYROGENIC STEROIDS IN ANIMALS*

By

R. H. Palmer

Several 5 β -H steroids produce intense fever and inflammation when injected into man.¹⁻³ The pyrogenicity of neutral steroids, such as etiocholanolone and 11-ketopregnanolone, is highly species-specific.⁴ This investigation was undertaken to determine whether the pyrogenicity of the acidic steroid lithocholic acid was similarly species specific, and to examine the possible inflammatory properties of the three steroids in experimental animals.

MATERIALS AND METHODS

Studies on inflammation were done on unrestrained animals. After shaving the abdomen, steroid solutions (20 mg/ml in propylene glycol) were injected subcutaneously on one side of the abdomen, and an equal volume of solvent vehicle on the other. Injection sites were observed for gross inflammation, and biopsies of steroid and control sites were taken at 48 or 72 hours from 4 animals at random in each steroid group.

RESULTS

Rabbits. Twelve rabbits each received 5 mg/kg (total dose 12.0 to 19.5 mg) and 0.5 mg/kg (1.2 to 2.0 mg) injections of lithocholic acid with corresponding controls. In 8 there was inflammation at the steroid but not control sites; in 3 there was inflammation at the steroid sites and minimal inflammation at the control sites; and in 1 there was inflammation at the steroid sites but more intense inflammation at the larger control injection site.

Twelve rabbits received 5 mg/kg (10.0 to 18.5mg) and 0.5 mg/kg (1.0 to 1.7 mg) injections of 11-ketopregnanolone with corresponding controls. The smaller doses produced no significant inflammation. In 3 animals there was inflammation at the 5 mg/kg site only; in 2 there was more inflammation at the 5 mg/kg site than at the corresponding control site; in 2 there was equivalent inflammation at the 5 mg/kg and control sites; in 1 there was greater inflammation at the control than at the 5 mg/kg site; in 2 there was inflammation only at the larger control site; and there was no inflammation at either site in 2 animals.

Twelve rabbits received 5 mg/kg (11.5 to 18.5 mg) and 0.5 mg/kg (1.2 to 1.9 mg) injections of etiocholanolone with controls. In 4 inflammation occurred only at one or both steroid sites; in 2 inflammation was greater at the steroid than at the control sites; in 2 there was equivalent inflammation at steroid control sites; in 1 there was inflammation only at the control site; there was no inflammation in 3 animals.

Guinea pigs. Five guinea pigs received 5 mg/kg (1.8 mg) injections of lithocholic acid; three showed inflammation at the steroid injection site, and 2 had no inflammation. Five guinea pigs received 5 mg injections; 2 showed inflammation at both steroid and control sites, and 3 showed no inflammation.

Five guinea pigs received 5 mg/kg (1.8mg) injections of 11-ketopregnanolone and 5 received

* This paper appeared in Proc. Soc. Exptl. Biol. Med., 119:108, 1965.

5 mg/kg injections. No significant inflammation was observed in any animal.

Five guinea pigs received 5 mg/kg (1.8 mg) injections of etiocholanolone; 2 had inflammation at the steroid site and 3 had no inflammation. Five guinea pigs received 5 mg injections; 4 had inflammation at the steroid site and 1 had inflammation at both steroid and control sites.

Hamsters. Five hamsters received 5 mg/kg (0.6 mg) injections of lithocholic acid; no inflammation was observed. Five hamsters received 5 mg injections; 1 had inflammation at the steroid site, 1 had no inflammation, and 3 died.

Five hamsters received 5 mg/kg (0.6 mg) injections of 11-ketopregnanolone; 1 had inflammation at the steroid site, 1 had much greater inflammation at the steroid than the control site, and 3 had no inflammation. Five hamsters had 5 mg injections; 1 had inflammation at the steroid site, 3 had inflammation at both steroid and control sites and 1 had inflammation at the control site.

Five hamsters received 5 mg/kg (0.6 mg) injections of etiocholanolone; 1 had inflammation at the steroid site and 4 had no inflammation. Five hamsters received 5 mg injections; 1 had inflammation at the steroid site, 1 had no inflammation and 3 died.

Rats. Five rats received 5 mg/kg (1.5 mg) injections of lithocholic acid and 5 received 5 mg injections. Three of the former did not have inflammation; all of the remaining animals showed mild inflammation at both steroid and control sites. Histologically, however, the inflammation was much more pronounced at the steroid sites than at the control sites.

Five rats received 5 mg/kg (1.5 mg) and 5 received 5 mg injections of 11-ketopregnanolone. Similar groups received the same doses of etiocholanolone. In no instance was there either gross or microscopic inflammation.

Dogs. Six dogs received 5 mg/kg (25 mg) injections of 11-ketopregnanolone and 48 hours later biopsies were obtained. This was repeated with etiocholanolone and lithocholic acid in each animal at the same doses. Gross inflammation was not observed with injections of 11-ketopregnanolone and etiocholanolone, but acute inflammation was demonstrable histologically at 3 of the six 11-ketopregnanolone sites and 4 of the 6 etiocholanolone sites. Intense inflammation, manifested by large (4 x 4 cm) areas of abscess formation, resulted from 4 of the 6 injections of lithocholic acid. No changes were seen at the remaining sites.

Histological changes. Histologically similar inflammation was produced by the 3 steroids at 24 and 48 hours in all species examined. Intense focal inflammation was present in some areas, with degenerating muscle fibers, thrombosed blood vessels, and sterile abscess formation. In other areas, there was a diffuse infiltration of round cells and polymorphonuclear leukocytes (Figure 1-A and D). Control injection sites usually showed no changes, but when steroid inflammation was particularly intense, diffuse edema with some round cell infiltration was seen (Figure 1-B). Biopsies from animals with inflammation at both steroid and control sites generally showed more severe and more acute inflammation at the steroid site, as shown in Figure 1-A and B. Inflammation produced by the smaller steroid dose was similar to but less intense than that produced by the larger steroid dose.

Temperature studies. Lithocholic acid, 5mg/kg, was injected intramuscularly in 8 rabbits, 5 rats, 4 dogs, 4 cats and 5 monkeys. Rectal temperatures were recorded continuously with a thermister probe as described previously.⁴ No temperature elevations were observed in any animal.

Figure 1. A. Intense inflammation produced by 5 mg of lithocholic acid injected subcutaneously in a rat. B. Moderate inflammation at the control site in the same rat. C. Absence of inflammation in a rat injected with 5 mg etiocholanolone. D. Inflammation produced by 5 mg of etiocholanolone in a guinea pig.

DISCUSSION

The results of the inflammation studies are summarized in Figure 2. Comparison of the number of animals showing inflammation only at the steroid site with the number showing inflammation only at the control site indicates that all of the steroids tested had significant inflammatory properties. There was considerable variation, however, in species and individual susceptibility to these inflammatory effects. For example, rabbits and hamsters developed inflammation from 11-ketopregnanolone while guinea pigs and rats did not; individual dogs tended either to respond to all 3 steroids or to none at all.

Since the ability of steroids to injure cells and produce inflammation may be related more to the total amount injected than to the amount per kg of body weight, half of the animals in each group received at least 5 mg of steroid. Even with this large dose, which produces intense fever and inflammation in most humans, all 5 guinea pigs responded to etiocholanolone with inflammation (see Figure 1-D) but only 2 of 5 responded to lithocholic acid. Conversely, the rats responded to lithocholic acid but not to etiocholanolone (see Figure 1-A and C). The factors responsible for these variations are unknown. It is possible that the variable order of hemolytic activity exhibited by different hemolysins against erythrocytes of several species⁵ may have a similar basis.

Figure 2. Inflammation produced by three representative steroids.

The role of the solvent vehicle in eliciting inflammation is not clear, but it seemed to be of more importance when inflammation was present elsewhere. For example, none of the injections in rats receiving neutral steroids produced inflammation, while all of the control sites in rats with lithocholic acid inflammation showed inflammatory changes (Figure 1-C and B). Furthermore, in all species, most of the animals with inflammation at both injection sites had much more intense inflammation at the steroid site. Therefore it seems possible that in some cases the changes at the control site may actually have been secondary in some way to the intense inflammation at the adjacent steroid injection site.

Lithocholic acid, like the neutral steroid pyrogens, failed to elicit fever in animals, and therefore it does not seem possible at present to study the mechanism of steroid pyrogen activity in species other than man. It is possible, however, to study the inflammatory and cytotoxic activity of steroids in animals, and such studies may be of importance in understanding certain effects of these substances, such as their ability to produce hemolysis,⁶ cirrhosis of the liver,⁷ and other phenomena.

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SUPPRESSION OF THE SHUNT PATHWAY IN PRIMARY GOUT BY AZATHIOPRINE*

By

L. B. Sorensen

An increasing volume of studies¹⁻³ lends support for a dual etiology of hyperuricemia in primary gout. In this disorder there may be either normal or excessive production of uric acid. Patients with primary gout related to overproduction of uric acid show a pattern of incorporation of a labeled precursor (e.g., glycine) into urinary uric acid which consists of a high initial value followed by a fairly rapid decline in isotope concentration during the succeeding days (Figure 1).

Figure 1. Concentration of ^{14}C in urinary acid following injection of glycine-1- ^{14}C into a normal subject and a gouty subject with overproduction of uric acid.

This pattern has been taken as evidence of a shunt pathway whereby precursor is incorporated into uric acid more promptly than in normal man by bypassing nucleic acid purines. This shunt pathway is generally considered to be responsible for overproduction of uric acid in primary gout.

When azathioprine (Imuran) was given to gout patients who displayed excessive excretion of uric acid in the urine, we found a significant reduction in both plasma and urinary uric acid. To evaluate this finding more precisely, the incorporation of glycine into uric acid during treatment

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with azathioprine was studied in three patients who had previously been shown to possess the shunt pathway.

The administration of azathioprine to patients with primary gout was an indirect result of a joint study with Drs. N. Bricker and R. Rieselbach at Washington University, St. Louis, on the nature of primary gout in a patient who had developed progressive renal failure due to gouty nephropathy which necessitated renal homotransplantation in December, 1964.⁴ The patient has, since then, been maintained on azathioprine in order to suppress a homograft reaction.

EXPERIMENTAL

Three gouty subjects previously shown to be over-producers of uric acid were studied in detail. In addition, plasma and urinary uric acid were determined daily in one patient with primary gout not related to overproduction of uric acid, and in two healthy individuals. Each subject received azathioprine* for a period of from 7 to 10 days in a daily dose of 300 or 400 mg (3.4 - 4.6 mg per kg body weight). No depressive effect on the bone marrow, nor any other untoward reaction was observed as a consequence of this treatment.

For three days before as well as during the study the patients were maintained on a diet essentially free of purines, but with sufficient protein to maintain nitrogen balance. Plasma and urinary uric acid were determined by an enzymatic spectrophotometric method.⁵ Glycine-1-¹⁴C was injected intravenously in a dose of approximately 100 μ c. In studies on the effect of azathioprine upon uric acid metabolism, glycine-¹⁴C was given 48 or 72 hours after initiation of drug treatment. Uric acid was isolated from urine and purified as described elsewhere,⁶ except that the first step in the isolation procedure involved either the addition of 200 mg of uric acid in solution as carrier to 200 ml of urine, or the adsorption of urinary uric acid to charcoal (Darco G-60) and its subsequent elution with hot 0.1 N NaOH.

Radioassay of ¹⁴C was done either in a vibrating reed electrometer after wet oxidation of uric acid in a vacuum combustion line, or in a liquid scintillation spectrometer (Packard Tri-Carb, Model 3324) using the following technic: preweighed amounts of uric acid (usually less than 50 mg) were dissolved in 2 cc of hyamine hydroxide, then mixed with 18 cc of a mixture of PPO (2,5-diphenyloxazole) 3 g, POPOP (1,4-bis-2-(5-phenyloxazolyl)-benzene) 100 mg, and toluene 1000 cc. In this system toluene-¹⁴C counted with an efficiency of about 70 per cent. In counting uric acid a modest quenching was observed which was proportional to the quantity of uric acid counted; (a sample weighing 50 mg reduced the counting efficiency to 85 per cent of the efficiency at zero weight). All samples were corrected for quenching either by including internal standards or by applying a factor derived from a curve relating degree of quenching to sample weight.

The miscible pool and turnover of uric acid were determined as described elsewhere,⁶ except that radioassay of ¹⁴C was done in a vibrating reed electrometer.

RESULTS

The effect of azathioprine on plasma and urinary uric acid in one of the gouty subjects with overproduction of uric acid is graphically presented in Figure 2. The patient had been maintained on a uricosuric drug (sulfipyrazone 600 mg daily) for two years and this treatment was continued

*Kindly supplied by Dr. P. Mawdsley, Burroughs, Wellcome and Company, Tuckahoe, New York.

Figure 2. Effect of azathioprine (Imuran) on plasma and urinary uric acid in a patient with primary gout. Throughout the study he was also on a uricosuric drug.

throughout the study. With addition of azathioprine, plasma uric acid fell from a pretreatment level of between 4.42 and 4.80 mg per cent to a low of 2.13 mg per cent on the 10th day, while the 24-hour urinary uric acid excretion fell from a high of 848 mg to a low of 367 mg. Seven days after discontinuation of the drug, plasma and urinary uric acid had returned to pretreatment levels. No significant changes in plasma and urinary uric acid were observed when azathioprine was given to two normal individuals and to a gouty patient whose hyperuricemia was related to a dysfunction in the enzymatic renal tubular transport mechanism of uric acid.

Figure 3 shows the incorporation of labeled glycine into urinary uric acid in three gouty subjects with excessive urinary uric acid excretion. The data of the upper curves were obtained when the patients were untreated, and clearly demonstrate the presence of the shunt pathway. The lower curves represent the data obtained when the patients had been pretreated with azathioprine for from 48 to 72 hours before injection of glycine- ^{14}C . With the exception of an abortive early peak in one of the patients, there was a striking conversion of the incorporation pattern to normal in all three patients. Patient J. L. was restudied 72 hours after azathioprine had been discontinued: The glycine incorporation curve at this time indicated a fractional return of the direct pathway of uric acid synthesis.

Table 1 summarizes all data on the cumulative incorporation of glycine into urinary uric acid, data on miscible pool turnover of uric acid, as well as pertinent clinical information.

DISCUSSION

The results in the preceding section indicate that when azathioprine is given to gouty subjects with overproduction of uric acid, urinary uric acid falls to within the normal range. Furthermore, the incorporation of precursor glycine into urinary uric acid reverts to a normal pattern, and the cumulative recovery of glycine- ^{14}C in urinary uric acid assumes values comparable to

GLYCINE INCORPORATION INTO URINARY URIC ACID

Figure 3. Concentration of ^{14}C in urinary uric acid following intravenous injection of glycine-1- ^{14}C at zero time into three patients with primary gout associated with overproduction of uric acid. All data are adjusted to a dose of $100\text{ }\mu\text{c}$ of glycine- ^{14}C . The lower curves represent data obtained after pretreatment with azathioprine for 48 to 72 hours. Patient J. L. was re-injected with glycine- ^{14}C on the 10th day, 72 hours after azathioprine had been discontinued.

those obtained in normal individuals. The exact site of action of azathioprine upon purine biosynthesis is not yet known, but work is currently underway in this laboratory to elucidate the precise locus of interaction.

There is growing evidence for the view that the most important mechanism for the precise control of a biosynthetic pathway is feedback inhibition. Figure 4 depicts a simplified scheme of the formation of uric acid. The first step in purine biosynthesis is the enzymatic reaction between glutamine and 5-phosphoribosyl-1-pyrophosphate to yield 5-phosphoribosylamine. The reaction generating phosphoribosylamine is irreversible and, therefore, becomes the rate-limiting reaction in de novo purine biosynthesis. Once formed, phosphoribosylamine will undergo a series of reactions to yield inosine-5'-phosphate (IMP), the parent purine compound. Most of the IMP is converted to adenylic acid (AMP) and guanylic acid (GMP), which are then incorporated into more complex polynucleotides. Even in the non-gouty subject a small part of inosinic acid appears to be degraded by a more direct pathway to uric acid. It is an increase of this latter pathway that characterizes primary gout with overproduction of uric acid.

The exact biochemical defect responsible for the exaggerated shunt pathway in primary gout is unknown. Factors governing the rate of synthesis of 5-phosphoribosylamine include the concentrations of the substrates glutamine and 5-phosphoribosyl-1-pyrophosphate, and the activity of the synthesizing enzyme. Wyngaarden and associates⁷ have been primarily interested in the regulation of the first enzyme of purine biosynthesis. They found that this enzyme had at least two separate regulatory sites, one for adenylyl and one for guanylyl ribonucleotides. The conversion of IMP to AMP and IMP to GMP is also controlled by feedback inhibition. Recently Gutman and Yü⁸ have suggested that the increased purine synthesis of primary gout may be a consequence of a defect in utilization of glutamine for renal production of ammonia, and as a result, extra glutamine is

Table 1

MISCIBLE POOL AND DAILY TURNOVER OF URIC ACID, AND CUMULATIVE EXCRETION OF ^{14}C IN URINARY URIC ACID FOLLOWING INTRAVENOUS ADMINISTRATION OF GLYCINE-1- ^{14}C BEFORE AND DURING TREATMENT WITH AZATHIOPRINE TO THREE PATIENTS WITH PRIMARY GOUT

Case	Age; weight	Presence of tophi	Miscible pool mg	Turnover mg/day	Dose of azathioprine	Other medication		Cumulative recovery of glycine- ^{14}C in urinary uric acid (7 days) per cent of dose	
						Control study	Azathioprine study	Control study	Azathioprine study
C.C.	28 yrs 118 kg	None	2,907	1,203	400 mg daily x 7	None	None	0.406	0.087
A.D.	51 yrs 79 kg	Extensive	2,397	1,185	300 mg daily x 7	None	Sulfinpyrazone 600 mg daily	0.521	0.284
J.L.	66 yrs 65 kg	None	2,821	1,251	300 mg daily x 10	None	Sulfinpyrazone 800 mg daily	0.512	0.101

Figure 4. Simplified scheme of the formation of uric acid.

diverted into the first and rate-determining reaction of de novo purine biosynthesis.

In studies with azathioprine the specific activity of ^{14}C in urinary uric acid (DPM of ^{14}C per mg uric acid) after glycine- ^{14}C administration was similar to that observed in normal subjects, which suggests that azathioprine does not suppress the synthesis of adenylic and guanylic acids and their incorporation into nucleic acids. Additional evidence in support of this view is provided by the finding that azathioprine had no effect upon uric acid metabolism in normal subjects.

The possibility cannot be excluded that the functional activity of the direct pathway of uric acid synthesis from IMP is affected by regulatory factors, and furthermore, that the biochemical error in primary gout with overproduction of uric acid may be related to a defect in the rate control of the shunt pathway which, in turn, becomes the driving force on the rate of synthesis of 5-phosphoribosylamine.

The potential usage of azathioprine in the treatment of primary gout related to overproduction of uric acid must await further experiments designed to establish the dose range that will effectively lower the production of uric acid.

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THE ERYTHROPIETIC RESPONSE TO TESTOSTERONE IN MALE AND FEMALE MICE*

By

W. Fried[†] and C. W. Gurney

The fact that pharmacological doses of testosterone stimulate erythropoiesis in female mice,¹ castrated rodents,² and hypophysectomized rodents³ has been repeatedly demonstrated. That testosterone in physiological doses, is also capable of influencing erythropoiesis in these animals is suggested by the observed differences in the red cell parameters of males and females,⁴ and by the drop in red cell counts following castration.² The present paper describes the differences in the response of male and female mice to testosterone, polycythemia, and hypoxia; discusses possible reasons for these differences; and considers their implications in regard to the physiologic role of testosterone in the regulation of erythropoiesis.

METHODS

Seven to ten week old male and female CBA mice were used throughout the study. Testosterone was commercially prepared as testosterone propionate in sesame oil at a concentration of 25 mg per cc.

Orchiectomies were performed via a small scrotal incision, and oophorectomies by a single ventral incision. Sham operated mice were identically incised, the ovaries or testes exposed, then replaced before wound closure. All animals were anesthetized with diabutal during surgery.

Polycythemia was induced by two daily intravenous injections of 0.5 cc packed RBC's unless otherwise indicated. The effect of testosterone was studied by injecting 2.5 mg of testosterone propionate subcutaneously on each of the two days of transfusion. One-half microcurie of ⁵⁹Fe was given 96 hours after the second injection of testosterone, and the 72 hour incorporation of radioiron into newly formed erythrocytes was determined, and calculated as a percentage of the injected dose of iron. A blood volume of 7 per cent of the body weight was selected for the calculation. Since in our experience the blood volume of normal mice is 5.5 per cent of body weight and that of mice receiving 2.0 cc of packed RBC's is 7.0 per cent of the body weight, there is actually an error of as much as 20 per cent when mice are transfused with smaller volumes of blood, as in the experiment illustrated in Figure 1. In this experiment, however, we are primarily interested in comparing male and female response and since this error pertains equally to both, we did not determine the blood volume for each of the groups.

Hypoxia was induced by maintaining the mice for 8 hours at a pressure of 60 per cent of normal atmospheric pressure in a chamber of the type described by Cotes and Bangham.⁵ Immediately after removal from the chamber, the mice were exsanguinated and the plasma was

* This report is taken from a paper that appeared in J. Lab. and Clin. Med., 67:420, 1966.

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Figure 1. The effect of testosterone in male and female mice with various degrees of plethora. () represents number of mice. I represents $\pm$ one standard error.

assayed for erythropoietin according to the method of DeGowin, Hofstra, and Gurney.⁶

X-radiation was administered by a 250,000 volt deep x-ray unit with 0.25 mm cu and 1.0 mm Al filtration; dose rate factor 57 R per minute; target distance 81 cm.

Bone marrow cells were obtained from the femurs of male and female CBA mice as follows: Mice were killed by transection of the cervical cord, the femurs were then disjointed and their heads transected. A number 22 disposable needle was introduced into the distal end of the medullary cavity, the proximal end of the femur was immersed in Tyrode's solution, and the Tyrode's solution was slowly flushed through the medullary cavity. The marrow collected in this manner from 10 mice was pooled and suspended in Tyrode's solution. A nucleated cell count was performed and the volume adjusted to a concentration of 10^7 nucleated cells per 0.55 cc with Tyrode's solution. Five-tenths cubic centimeter of this suspension was then injected intravenously into female mice after 900 R total body x-radiation.

RESULTS

The effect of testosterone on erythropoiesis in male and female polycythemic mice. Male and female CBA mice were made polycythemic by injection with the volumes of blood indicated in Figure 1. This figure also shows the 72 hour iron incorporation expressed as a percentage of the injected dose. It can be seen that both sexes respond to testosterone less markedly as the degree of plethora increases. The rate of erythropoiesis is less depressed in control males than it is in control females. Thus, even after transfusing 1.6 cc of cells, which is more than twice the animal's red cell mass, the rate of iron incorporation of normal (control) male mice remains at 2.4 per cent. The rate of iron incorporation of female mice is consistently lower than 1 per cent after transfusion with only one-half of this volume. In every case testosterone stimulates the rate of erythropoiesis of female mice to a greater extent than that of males.

The effect of testosterone on erythropoiesis in lethally irradiated female mice injected with male or female bone marrow cells. Female mice received 900 R total body x-radiation

and immediately afterwards were injected with a suspension containing 10^7 bone marrow cells from either male or female CBA mice. Two and five-tenths milligrams of testosterone was injected into the recipients on days 0, 1, and 2 after x-irradiation. Five-tenths cubic centimeter packed red blood cells was injected on days 1 and 2 after irradiation. Iron-59 was injected on day 6, and the 72 hour incorporation was determined and is recorded in Figure 2. The figure indicates that male bone marrow cells, when transplanted into an irradiated female host, respond to testosterone even more vigorously than do female cells, and are suppressed by polycythemia to the same extent as are female cells.

Figure 2. The effect of testosterone on iron incorporation of lethally x-irradiated female mice receiving transplants of male or female bone marrow. () represents number of mice. [represents $\pm$ one standard error.

The effect of orchietomy or oophorectomy on the erythropoietic response to testosterone. The effect of testosterone on erythropoiesis in male and female mice 1 month and 4 months after orchietomy or oophorectomy is shown in Figures 3 and 4. One month after surgery, neither orchietomized males nor oophorectomized females differ from sham operated mice in their response to testosterone. After 4 months, however, the response of orchietomized males to testosterone is more than 3 times greater than the response of sham operated males. The response of oophorectomized females at 4 months does not differ from the response of sham operated females.

Erythropoietin titers in the plasma of male and female mice treated with testosterone and subjected to hypoxia. Male and female mice received 2.5 mg of testosterone or 0.1 cc of sesame oil on two consecutive days. Forty hours after the second injection, some of them were placed in an environment maintained at 60 per cent atmospheric pressure for 8 hours. These mice were then killed and exsanguinated, and their plasma was assayed for erythropoietic activity. Other mice receiving identical injections but not subjected to hypoxia were exsanguinated, and the plasma was collected 48 hours after the final injection of testosterone. The results are shown in Figure 5, which records the percentage of iron incorporation of assay mice after re-

Figure 3. The effect of orchiectomy on the erythropoietic response to testosterone. () represents number of mice. [represents $\pm$ one standard error.

Figure 4. The effect of oophorectomy on the erythropoietic response to testosterone. () represents number of mice. [represents $\pm$ one standard error.

ceiving plasma from mice treated as indicated in the legends to Figure 3 and 4. The plasma of male mice contains more erythropoietin after hypoxia than does the plasma of female mice. On the other hand, the erythropoietin titer of female mice treated with testosterone before hypoxia is more than twice as high as that of similarly treated males.

DISCUSSION

The data presented here indicate that erythropoiesis is less easily suppressed by polycythemia in male mice than it is in female mice, and that the plasma of male mice subjected to hy-

Figure 5. Erythropoietin levels in plasma of male and female mice treated with testosterone and hypoxia. () represents number of mice. I represents $\pm$ one standard error.

poxia contains higher erythropoietin titers than does the plasma of female mice. Previously reported studies have accumulated evidence supporting the thesis that testosterone, when given to mice in pharmacological doses, will stimulate erythropoiesis,¹⁻³ and that this action is due, at least partially, to an increase in erythropoietin production.^{7,8} The differences reported here between the male and female response to hypoxia and polycythemia may then be interpreted as indicating that males, having a higher production of endogenous testosterone, produce more erythropoietin in response to any particular hypoxic stimulus.

In addition, we have shown that the rate of erythropoiesis and erythropoietin production are increased to a greater extent in females than in males following testosterone injection; and that this increased sensitivity to exogenous testosterone does not result from changes in the bone marrow cell itself. One month after castration, male mice are still less sensitive to testosterone than are females, but 4 months after castration, males are as sensitive as are females. Since testosterone titers fall shortly after castration, it is unlikely that more circulating endogenous testosterone per se accounts for the decreased sensitivity of male mice. Moreover, since female mice do not become less sensitive even 4 months after oophorectomy, it is unlikely that some ovarian function renders them more sensitive to the erythropoietic effect of testosterone.

The most probable interpretation of the data presented is that testosterone alters some organ other than the bone marrow with the effect of increasing erythropoiesis, and that this alteration regresses only slowly after the stimulus is removed. Since it has been shown that testosterone can stimulate erythropoietin production, and since we have here presented evidence consistent with the thesis that male mice have higher endogenous erythropoietin production, it is probable that testosterone acts upon the site of erythropoietin production. According to current concepts, this is the kidney, since this organ is known to be responsible for most of the erythropoietin production.⁹

We have previously presented evidence that testosterone has no erythropoietic effect in the

absence of the kidney.¹⁰ We therefore suggest that testosterone stimulates the kidney to increase the production of erythropoietin at any level of hypoxic stimulation, and that the constantly elevated endogenous titers of males render the kidney less sensitive to further alteration by injections of exogenous testosterone. It is possible that after castration it may take up to 4 months for the altered kidney to revert to an unstimulated state comparable to the state normally obtaining in the female. The studies of Korenchevsky and Selye and Kochakian have demonstrated the effect of testosterone on normal male,¹¹ female,¹² and castrated male kidneys.¹³ The kidneys of normal males, which are larger than those of females, can be reduced by castration to the size of the female kidney.¹³ This change takes 30 days if castration is performed before puberty.¹³ Since some androgenic effects subside more slowly and less completely when orchiectomy is performed post-puberty,¹⁴ it is not unreasonable to postulate that regression of the kidney size may also occur more slowly. Furthermore, the kidney of the normal adult female responds to testosterone with a rather marked increase in size, proliferation of the convoluted tubules, and alteration of the parietal epithelium of Bowman's capsule. The changes are less marked when testosterone is given to normal males, and are easily demonstrated in castrated males.¹¹ These studies support the thesis we have presented here. We are currently, however, accumulating more direct evidence correlating structural changes in the kidney to responsiveness to hypoxia, polycythemia, and testosterone.

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COMBINED EFFECT OF COBALT AND TESTOSTERONE ON ERYTHROPOIESIS*

By

W. E. Janda, W. Fried,[†] and C. W. Gurney

It has long been known that cobalt increases erythropoiesis in man and experimental animals,¹⁻⁴ and that it achieves its erythropoietic effect by initiating erythropoietin formation.⁵⁻⁶ The clinical effectiveness of testosterone therapy in various refractory anemias has led to investigations which suggest that one method by which this hormone promotes erythropoiesis is also by stimulating the elaboration of erythropoietin.⁷ The present report deals with the combined effects of cobalt and testosterone on erythropoiesis in transfusion-induced polycythemic mice, and on plasma erythropoietin levels in normal mice.

METHODS

All experiments were carried out on CF No. 1 female mice, 6 to 10 weeks of age and weighing 20-30 g.

Polycythemia was induced according to the method of DeGowin et al.⁸ by intravenous injection of an 80 per cent suspension of washed mouse RBC's on 2 consecutive days. All polycythemic mice had hematocrits between 55-75 per cent at the end of the experiment.

Testosterone was administered subcutaneously in the form of testosterone propionate in sesame oil in a concentration of 25 mg/ml. Cobalt was administered subcutaneously in the form of cobaltous chloride in normal saline from stock solution in a concentration of 25 μ moles/ml.

The rate of ⁵⁹Fe uptake into newly formed RBC's was determined as follows: One-half (0.5) μ c of ⁵⁹Fe chloride was given intravenously 60 hours after cobalt injection. Three days after injection of ⁵⁹Fe, the animals were killed and 0.25 ml of blood was removed by cardiac puncture. Radioactivity was measured in a well-type scintillation counter, and the percentage of the injected dose of ⁵⁹Fe appearing in the peripheral red cells was calculated from these counts.⁸ Plasma erythropoietin titers were determined by the method of DeGowin et al.⁸

RESULTS

Erythropoietic effect of testosterone and varied doses of cobalt. On day 1 three groups of mice received single injections of 2.5 mg of testosterone in oil, while the appropriate controls received single injections of 0.1 ml of sesame oil. Sixty hours later, on day 3, single injections of either 1.0 or 2.5 μ moles of cobalt or normal saline were given to groups of experimental and control animals, as indicated in Figure 1. ⁵⁹Fe was given 60 hours after the injection of saline or cobalt. Polycythemia was induced by transfusing 0.7 ml RBC's on 2 consecutive days, the first injection being given on the same day as testosterone or sesame oil administration. Figure 1 shows that 2.5 μ moles of cobalt given alone will increase the rate of iron incorporation to 4

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Figure 1. Erythropoietic effect of testosterone and varied doses of cobalt.

times that in the saline controls. Two and five-tenths milligrams of testosterone, given 60 hours before either 1.0 or 2.5 μ moles of cobalt, will increase the rate of iron incorporation to 3 and 5 times, respectively, the rate in the corresponding sesame oil-cobalt controls.

Erythropoietic effect of cobalt and testosterone given at varied time intervals. Mice in groups IA and IB to VA and VB received single injections of 2.5 mg of testosterone on days 1 to 5. Control groups IX and IY to VX and VY received single injections of 0.1 ml of sesame oil on the same days. Subgroups IA to VA and IX to VX received single injections of 2.5 μ moles of cobalt in normal saline on day 5, while subgroups IB to VB and IY to VY received single injections of 0.2 ml of normal saline on this day. ⁵⁹Fe was given 60 hours later on day 8. Polycythemia was induced by transfusion with 0.55 ml RBC's 5 and 6 days before injections of ⁵⁹Fe.

Table 1 shows that when testosterone is given 0 to 4 days before cobalt, it exerts a synergistic effect on the rate of iron incorporation as compared to the control rate. Peak synergism is observed when testosterone is given 2 days or 3 days before cobalt.

Effect of testosterone and cobalt on plasma erythropoietin levels of normal mice. Two groups of mice received single injections of 2.5 mg of testosterone in oil, and their appropriate controls received single injections of 0.1 ml of sesame oil on day 1. On day 3, single injections of 2.5 μ moles of cobalt or normal saline were given to one of the 2 groups of experimental animals and to one of the two groups of controls, as indicated in Figure 2. Twelve hours later, on

Table 1
ERYTHROPOIETIC EFFECT OF COBALT AND TESTOSTERONE GIVEN AT
VARIED TIME INTERVALS IN TRANSFUSION-INDUCED
POLYCYTHEMIC CF NO. 1 FEMALE MICE

Group	No. of mice per group			% ⁵⁹ Fe uptake (S.E.)
I. A.	5	Testosterone, 2.5 mg (-4 days)	Cobalt, 2.5 μ M	1.6 $\pm$ 0.4
B.	8	Testosterone, 2.5 mg (-4 days)	Normal saline	0.5 $\pm$ 0.1
X.	7	Sesame oil	Cobalt, 2.5 μ M	0.4 $\pm$ 0.1
Y.	7	Sesame oil	Normal saline	0.3 $\pm$ 0.1
II. A.	8	Testosterone, 2.5 mg (-3 days)	Cobalt, 2.5 μ M	6.8 $\pm$ 1.4
B.	7	Testosterone, 2.5 mg (-3 days)	Normal saline	0.9 $\pm$ 0.3
X.	7	Sesame oil	Cobalt, 2.5 μ M	0.7 $\pm$ 0.2
Y.	7	Sesame oil	Normal saline	0.4 $\pm$ 0.0
III. A.	7	Testosterone, 2.5 mg (-2 days)	Cobalt, 2.5 μ M	7.0 $\pm$ 2.0
B.	5	Testosterone, 2.5 mg (-2 days)	Normal saline	1.0 $\pm$ 0.5
X.	7	Sesame oil	Cobalt, 2.5 μ M	0.5 $\pm$ 0.1
Y.	5	Sesame oil	Normal saline	0.4 $\pm$ 0.1
IV. A.	7	Testosterone, 2.5 mg (-1 day)	Cobalt, 2.5 μ M	4.5 $\pm$ 1.7
B.	6	Testosterone, 2.5 mg (-1 day)	Normal saline	1.2 $\pm$ 0.4
X.	7	Sesame oil	Cobalt, 2.5 μ M	0.7 $\pm$ 0.2
Y.	7	Sesame oil	Normal saline	0.5 $\pm$ 0.0
V. A.	7	Testosterone, 2.5 mg (0)	Cobalt, 2.5 μ M	3.2 $\pm$ 1.0
B.	7	Testosterone, 2.5 mg (0)	Normal saline	0.5 $\pm$ 0.1
X.	7	Sesame oil	Cobalt, 2.5 μ M	0.6 $\pm$ 0.1
Y.	6	Sesame oil	Normal saline	0.3 $\pm$ 0.1

() Days before cobalt injection.

day 4, all the animals were killed and their plasma was assayed for erythropoietic activity.

Figure 2 shows that plasma from normal mice receiving either 2.5 mg of testosterone or 2.5 μ moles of cobalt alone will produce respectively, one and one-half- and 2-fold increments in the rate of iron incorporation compared to plasma from mice receiving saline and sesame oil. Plasma from mice receiving both testosterone and cobalt produces a 14-fold increase in the rate of iron incorporation over control values, which is substantially more than would be expected from the summed effects of testosterone or cobalt given separately.

DISCUSSION

Cobaltous chloride, given orally or parenterally for a period of weeks, induces and maintains a polycythemia in man and experimental animals.²⁻⁴ This erythropoietic effect is exerted through increased production of erythropoietin.^{5,6} Testosterone increases erythropoiesis in various experimental animals⁹⁻¹¹ and has been used effectively to treat some previously refractory anemias.¹²⁻¹⁴ Evidence has been presented suggesting that one way in which testos-

Figure 2. Effects of testosterone and cobalt on plasma erythropoietin levels.

terone affects erythropoiesis is also by stimulating the elaboration of erythropoietin.⁷

In the experiments reported here, 2.5 mg of testosterone produced a minimal or no erythropoietic effect. This is probably attributable, in the first experiment, to the fact that larger volumes of blood (and hence, greater red cell masses) were used than in subsequent experiments. It has been demonstrated in the preceding paper that excessive plethora dampens the erythropoietic effect of testosterone.¹⁵ In the first experiment it was noted that 2.5 μ moles of cobalt alone produced an increased response. This dose was therefore used in the succeeding experiments.

The first and second experiments show that cobalt and testosterone in combination act synergistically in increasing the rate of iron incorporation into the red cells of polycythemic mice. This finding was interpreted as demonstrating that cobalt and testosterone in combination potentiate erythropoiesis. That peak synergism occurs when testosterone is given 2 days or 3 days before cobalt is consistent with previous findings that peak erythropoietin formation is reached 12 hours after injection of cobalt and 3 days after injection of testosterone.^{6,7}

The third experiment indicates that cobalt and testosterone in combination act synergistically to increase erythropoietin levels in the plasma of normal mice. It is of interest to note that the increase in erythropoietin levels observed in experiment 3 is of the same order of magnitude as the increase in erythropoiesis measured in experiments 1 and 2. The third experiment reconfirms the hypothesis that both agents stimulate erythropoiesis via increased production of erythropoietin. The synergistic interaction demonstrated in these experiments, however, suggests

that the fundamental mechanisms by which cobalt and testosterone stimulate elaboration of erythropoietin are quite different. Investigation of these mechanisms awaits the elucidation of the biosynthetic pathway of erythropoietin action.

The clinical use of cobalt in cases of refractory anemias is limited by its toxic effects: The use of testosterone is also limited by its side effects, especially in female patients. These experiments suggest that greater clinical effectiveness, with fewer side effects, might be obtained by combining lower doses of both agents, than can be obtained with either medication alone.

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USE OF MILD PLETHORA TO DEMONSTRATE AN ERYTHROPOIETIC EFFECT FROM SMALL AMOUNTS OF ANDROGENS*

By

W. Fried,[†] and C. W. Gurney

On the basis of evidence presented in previous reports,¹⁻⁴ it has been postulated that testosterone may, at least in part, stimulate erythropoiesis by its effect on erythropoietin production. This implies that testosterone's effect on various refractory anemias may also be mediated by an increase in erythropoietin production. A frequent criticism of this conclusion has been that the doses of testosterone used were extremely large. We have recently reported that increasing degrees of plethora dampen the erythropoietic effect of testosterone.⁵ In the present paper we shall present evidence that small doses of testosterone can be shown to exert an erythropoietic effect.

METHODS

The mice were 10- to 12-week-old CF No. 1 females weighing 23-26 g. Testosterone propionate was diluted in sesame oil to provide the desired dose in a volume of 0.1 cc.

Mouse erythrocytes were collected, washed once and resuspended in saline in 80 per cent concentration. Polycythemia was induced by injection of the indicated volume of packed RBC's on each of two consecutive days. Testosterone was administered subcutaneously on the same days. One-half microcurie of radioiron was given intravenously 96 hours after the final injection of testosterone, and the 72 hour radioiron incorporation into newly formed erythrocytes was determined and expressed as a percentage of the injected dose.

Mice were made hypoxic by placing them for 8 hours in an evacuated chamber of the type described by Bangham and Cotes⁶ with the pressure at 60 per cent of atmospheric pressure. Erythropoietin titers were assayed as described by DeGowin et al.⁷

RESULTS

Effect of testosterone on mildly plethoric mice. Mild plethora was induced by injecting 0.2 cc of packed red cells daily for two consecutive days. Testosterone in the dosage indicated in Table 1 was injected on the same days as transfusion. Results indicate that two injections of as little as 0.1 mg of testosterone are sufficient to stimulate erythropoiesis in mildly plethoric mice, and the degree of erythropoietic stimulation increases as the dose increases. One milligram of testosterone increases erythropoiesis to more than 11 times the control value.

Effects of small doses of testosterone on the erythropoietin titers of hypoxic mice. Mice were injected with either 0.25 mg of testosterone or 0.1 cc of sesame oil on each of two consecutive days. Forty hours after the final injection, some of the animals were placed in an environ-

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Table 1

EFFECT OF SMALL DOSES OF TESTOSTERONE ON MILDLY PLETHORIC MICE

0.2 cc RBC x 2	No. of animals	Hematocrit	⁵⁹ Fe uptake $\pm$ S.E.
Sesame	7	47 ± 0.5	1.9 ± 0.3
0.1 mg testosterone	6	47 ± 1	4.5 ± 0.8
0.25 mg testosterone	8	47 ± 1	8.8 ± 2.1
0.50 mg testosterone	8	50 ± 1.6	11.7 ± 1.9
1.0 mg testosterone	7	48 ± 1.2	21.4 ± 3.2

ment maintained at 60 per cent atmospheric pressure for 8 hours. They were then exsanguinated and the plasma assayed for erythropoietin. The plasma of non-hypoxic mice was assayed for erythropoietin 48 hours after the second injection of testosterone or sesame oil. Figure 1 shows that the erythropoietin titers of hypoxic mice treated with 0.25 mg of testosterone are 4 times higher than those of hypoxic mice treated with sesame oil. Non-hypoxic mice treated with 0.25 mg of testosterone did not have detectably elevated erythropoietin titers.

Figure 1. The effect of testosterone and hypoxia on erythropoietin titers. () represent number of mice. I represents $\pm$ one standard error.

DISCUSSION

Testosterone stimulates erythropoiesis in polycythemic mice and does so, at least partially, by increasing erythropoietin production.^{3,4} A frequent objection raised against applying these studies to the interpretation of the effect of testosterone on human erythropoiesis has been that extremely large doses of testosterone have been employed.

Previously we have shown that the effect of testosterone on erythropoiesis is more marked

in mildly plethoric mice.⁵ Consequently, using only mildly plethoric mice, we have re-investigated the minimal dose of testosterone necessary to observe a detectable erythropoietic effect. We have shown here that as little as 0.1 mg of testosterone given on two consecutive days produces an increase in the erythropoietic rate. On a weight for weight basis this dose is comparable to doses administered in human disease. Since the effect of testosterone increases with decreasing plethora, one might expect that even smaller doses would affect the non-plethoric mouse, although their effects would be difficult to detect because they would be masked by the high basal rate of erythropoiesis.

The data also indicate that as little as 0.25 mg of testosterone produces measurable increases in the erythropoietin titers of hypoxic mice. It is thus unlikely that effects on erythropoiesis and erythropoietin formation are due only to some non-specific effect of extremely large doses of testosterone.

The above experiments also emphasize the value of mildly plethoric mice for the study of erythropoietic stimuli that are dependent on erythropoietin for their effect. Triiodothyronine,⁴ cobalt⁸ and testosterone all have a more profound effect on the rate of erythropoiesis of mildly plethoric mice than on mice made more intensely plethoric.

Although it may be premature to interpret the role of testosterone in the therapy of human anemias on the basis of these experiments in mice, the demonstration that doses of the hormone that are effective in the mouse are comparable to those used in the clinics, encourages us to investigate the effect of testosterone on erythropoietin levels in anemic patients.

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ON THE MECHANISM OF ERYTHROPOIETIN-INDUCED DIFFERENTIATION
III. THE NATURE OF ERYTHROPOIETIN ACTION ON ^{14}C
GLUCOSAMINE INCORPORATION BY MARROW
CELLS IN CULTURE*

By

P. P. Dukes and E. Goldwasser

In previous papers concerning the mechanism of action of erythropoietin on bone marrow cells in vitro,^{1,2} we have suggested that the hormone may act as a de-repressor of those genes controlling the synthesis of components characteristic of the differentiated erythroid cell. Erythropoietin would then act as a primary inducer of the process of erythrocyte development from stem cells. Similar proposals, based on the Jacob and Monod model of the regulation of bacterial gene activity³ have been made by various authors for other hormones.⁴⁻⁷ Here we will describe some studies of the erythropoietin-dependent incorporation of ^{14}C glucosamine into stroma by rat marrow cells in short-term culture. In this system the stimulated uptake of glucosamine seems to be of relatively short duration in each cell, resulting in a limited amount of labeled stroma per cell. This finding provides a formal analogy to the induction of enzyme synthesis in bacteria. A preliminary report on some parts of this paper has been published.⁸

MATERIALS AND METHODS

Bone marrow cells from male Sprague-Dawley rats were collected and incubated in the presence of 0.1 μC of 1- ^{14}C glucosamine (2.5 mC/mMole)[†] as described previously⁹ with the following modifications: the volume of the cultures was reduced to 1 ml, and incubations were performed in 35 x 10 mm plastic culture dishes[‡] in an atmosphere of 95 per cent air and 5 per cent CO_2 . In some later experiments the incubation medium was changed from a 1:1 mixture of 199[§] and calf serum[¶] to a 1:1 mixture of NCTC 109[§] and calf serum, which resulted in substantially better incorporation of glucosamine. After incubation the dishes were chilled for 10 min at 4°. The cell suspensions were washed over to centrifuge tubes, centrifuged for 5 min at 500 x g, resuspended in 4 ml saline, centrifuged again and resuspended in 2 ml saline.^{**} They were then collected on cellulose ester membranes of 0.45 μ mean pore size and 47 mm diameter^{††} by a procedure modified from Kahan,¹⁰ and washed twice with 2 ml of saline. To insure constant counting geometry the cell suspensions were always placed on a wetted central area of 23 mm diameter. The filters were air dried and counted on steel planchets. Because of their

* This paper appeared in Biochim. Biophys. Acta 108:447, 1965. ✓

† New England Nuclear Corporation.

‡ Falcon Plastics, Inc.

§ Microbiological Associates.

¶ Colorado Serum Company.

** We have recently found that the cell suspensions may be filtered directly, eliminating the prior washing, without altering the results.

†† Millipore HAWP 04700.

tendency to curl, the filters were held flat by steel rings of 3 mm annular width and 47 mm outside diameter. Samples were counted in a thin window, low background counter. No significant self-absorption was noted when samples containing up to $9 \cdot 10^6$ cells were counted on membrane filters.

RESULTS

The distribution of radioactivity. We have previously reported⁹ that stroma from marrow cells incubated with ^{14}C glucosamine, whether prepared by acid precipitation or by water lysis followed by centrifugation, contained about one-half of the total radioactivity found in the whole cells. About 30 per cent of the stroma label was found, after acid hydrolysis, in N-acetyl and N-glycolyl neuraminic acids; none was present as free glucosamine. To justify the use of data derived from counting whole cells on membrane filters, it was necessary to compare the relative amounts of label in the whole cells and in the insoluble stroma fraction from cultures incubated with different amounts of erythropoietin.* The data in Table 1A show that after 24 hours of incubation about 60 per cent of the total cellular radioactivity was found in the stroma at all dose levels of hormone. The slopes of the linear portion of the log-dose response curves were different for the two methods. The whole cells incorporated a greater increment of label per

Table 1

DISTRIBUTION OF RADIOACTIVITY BETWEEN SOLUBLE AND INSOLUBLE FRACTIONS OF MARROW CELLS

A. All samples contained $7 \cdot 10^6$ nucleated cells/ml in 50% 199, 50% calf serum and were incubated for 24 h. Whole cells were collected on filters; soluble and insoluble fractions were obtained from washed cells lysed in 1 ml of water by repeated freezing and thawing and separated by centrifugation. Values given are the means of quadruplicates. B and C. Samples contained $5.2 \cdot 10^6$ nucleated cells/ml in 50% NCTC 109, 50% calf serum and were incubated for either 6 or 24 h. Further treatment was as in A. Values given are the means of triplicates.

Expt.	Erythropoietin (units/ml)	Whole cells (counts/min)	Insoluble fraction (counts/min)	Soluble fraction (counts/min)	Radioactivity in insoluble fraction (%)
A 24 h	0	229	130	104	57
	0.05	272	166	120	61
	0.125	362	213	152	59
	0.25	443	270	196	61
	0.50	574	319	230	55
B 6 h	0	86	56		65
	0.20	136	92		67
C 24 h	0	720	401		56
	0.20	1276	776		61

* Erythropoietin (Step III, lot 137) was generously provided by the U. S. Public Health Service Study Section on Hematology.

unit of erythropoietin than did the stroma, suggesting that the hormone had a stimulatory effect on the soluble fraction of the cells containing glucosamine, or its derivatives, as well as on the insoluble stroma fraction. In another experiment (Table 1B and C) we compared the radioactivity in whole cells collected on filters with radioactivity in washed stroma from lysed cells, at 6 and 24 hours of incubation. The per cent of the total cell label found in the stroma fraction was slightly greater at 6 hours than at 24.

With the new method, the results are reported as total counts/min instead of as specific activity and it was essential, therefore, to culture the cells under as nearly identical conditions as possible. For this purpose, incubation in small plastic Petri dishes was found to be greatly superior to incubation in the tubes previously used. The linear portion of the log-dose response curve extended from about 0.1 unit to about 0.5 unit/ml under these conditions. The dose-response relationships at both 6 and 24 hours of incubation showed that the log-linear relationship holds at both times, with increased amounts of radioactivity incorporated, and therefore greater ease of measurement at the longer time.

We determined the duration of stimulated glucosamine incorporation after the cells had been exposed to erythropoietin for 1 hour. At this time the cells were transferred to medium that had been preincubated with cells in the absence of erythropoietin. The course of glucosamine incorporation was followed for the next 24 hours. The first set of controls for this experiment consisted of cells incubated for 1 hour in medium containing erythropoietin, centrifuged, and resuspended in the identical medium. The second set of controls consisted of cells treated in the same manner but omitting the erythropoietin.

The data in Figure 1 show that the rates of incorporation of the 2 erythropoietin-treated cultures were the same for the interval between 3 and 7 hours and were greater than in the cultures not containing the hormone. After the seventh hour of incubation, those cells that had been in contact with erythropoietin for only 1 hour showed a rate of glucosamine incorporation essentially the same as in the control without erythropoietin, while those cells that were in contact with the hormone for the total time maintained the original rate of incorporation.

The effect of inhibitors. Since the detailed mechanism of stroma biosynthesis is not known, it was important to determine whether protein synthesis is a necessary prerequisite for stimulated glucosamine incorporation. The data in Table 2 show that puromycin* caused a decrease of 95 per cent in glucosamine uptake by both control and erythropoietin containing cultures. No hormone effect was detectable in the presence of 50 μ g of the inhibitor.

Actinomycin D,[†] which inhibits the erythropoietin effect on hemoglobin synthesis² and on RNA synthesis,¹ also inhibits the stimulation of glucosamine incorporation due to the hormone (Table 3). The response to actinomycin added at the same time as erythropoietin was dependent on the ratio of amount of inhibitor to the number of cells. The erythropoietin effect was completely abolished at 0.25 μ g of actinomycin per million cells while at 0.156 μ g per million cells there was a short burst of stimulated incorporation which ceased after a few hours (Figure 2). For the first 4 hours of incubation there was no change in incorporation due to actinomycin, but in the next 3 hours the stimulated rate of incorporation was reduced by about 40 per cent in the presence of the inhibitor.

* Puromycin was bought from Nutritional Biochemicals Incorporated.

† Actinomycin D was a gift from Merck, Sharpe, and Dohme.

Figure 1. Duration of stimulated glucosamine incorporation by marrow cells after 1 hour exposure to erythropoietin. All cultures were begun with $4.2 \cdot 10^6$ nucleated cells/ml in NCTC 109: calf serum (1:1 v/v). Control (X) had no added erythropoietin, experimental curves (Δ and $\square$) had 0.12 units of erythropoietin/ml. After 1 hour of incubation cells were collected by centrifugation and resuspended in medium that had been preincubated in the presence of marrow cells for 1 hour. Medium for curves (X) and ($\square$) had no added erythropoietin while that for curve Δ had 0.12 units/ml. All samples had ^{14}C glucosamine added at 1 hour.

Table 2

EFFECT OF PUROMYCIN UPON ERYTHROPOIETIN-STIMULATED GLUCOSAMINE INCORPORATION

All cultures contained $4.4 \cdot 10^6$ nucleated cells/ml; incubation time was 24 h in medium containing NCTC 109, additions were made at zero time, values given are the means of 5 samples.

Additions	Counts/min
None	572
0.15 units of erythropoietin/ml	699
0.15 units of erythropoietin plus 50 μg of puromycin/ml	33

When actinomycin was added 15 hours after incubation had begun, the erythropoietin-stimulated glucosamine uptake was not affected for the next 3.5 hours, at which time incorporation ceased completely (Figure 3).

Table 3
INHIBITION OF ERYTHROPOIETIN EFFECT BY
ACTINOMYCIN D

All cultures contained $5 \cdot 10^6$ nucleated cells/ml and were incubated for 24 h; values given are the means of quadruplicates.

Erythropoietin (units/ml)	Control (counts/min)	Actinomycin D* (counts/min)
0	445	132
0.125	626	123
0.25	678	129
0.5	653	132

* 1.25 actinomycin/ml.

Figure 2. Effect of actinomycin D on glucosamine incorporation. Cultures were begun with $8 \cdot 10^6$ nucleated cells/ml in 199:calf serum (1:1 v/v). Erythropoietin (0.25 units/ml), actinomycin (1.25 μ g/ml) and 14 C glucosamine were all added at zero time. Curves labeled Δ and $\square$ had erythropoietin added, X and $\circ$ had no added erythropoietin; $\square$ and $\circ$ had actinomycin added.

Figure 3. Effect of actinomycin D on glucosamine incorporation after 15 hours of preincubation. All cultures were begun at $8 \cdot 10^6$ nucleated cells/ml. At zero time controls (X and ○) had no added erythropoietin, curves Δ and □ had 0.25 units/ml added. ^{14}C glucosamine was added to all cultures at zero time. At 15 hours (indicated by arrow) cultures ○ and □ had $1.25 \mu\text{g}$ actinomycin/ml added.

To test the possible role of cell division in erythropoietin action, cultures were incubated in the presence of 10^{-7} M colchicine.* The data in Table 4 show that colchicine had no effect on the erythropoietin-induced glucosamine uptake.

DISCUSSION

The use of a simplified procedure for determining the effect of erythropoietin upon the incorporation of glucosamine into marrow cells makes it possible to increase the number of samples and to work more rapidly. Although the fraction of the total cellular radioactivity represented by the stroma changes slightly with time, this change does not introduce any appreciable error in interpretation since the fraction becomes progressively smaller. In addition, the non-stroma fraction of the cells that is labeled with glucosamine seems to respond to the hormone, which makes it desirable not to discard that fraction when studying the action of erythropoietin.

When erythropoietin action on marrow cells in vitro is restricted, either by removal of the hormone or by addition of actinomycin D, the hormone-induced stimulation of glucosamine incorporation continues for 3 to 4 hours. Such a state of induced synthesis, which begins and ends abruptly, may be the result of what Kepes¹⁰ has termed "elementary waves." In the *Escherichia*

* Colchicine was purchased from Eli Lilly and Co.

Table 4

LACK OF EFFECT OF COLCHICINE UPON ERYTHROPOIETIN-
STIMULATED GLUCOSAMINE INCORPORATION

All cultures contained $6 \cdot 10^6$ nucleated cells/ml, incubation time was 23 h, additions were made at zero time, values given are the means of triplicates.

Additions	Counts/min
None	66
0.25 units of erythropoietin/ml	124
0.04 μ g colchicine/ml	59
0.25 units of erythropoietin plus 0.04 μ g of colchicine/ml	125

coli system they are waves of synthesis of B-galactosidase, while in the marrow cell system described here they are waves of induced incorporation of glucosamine.

In the bacterial system studied at constant inducer concentration, and in the marrow system studied at constant hormone concentration, there is a lag followed by a period in which the product accumulates at a rate that is linear with time.¹¹ We may infer that, in this latter period, the mRNA level corresponding to the product studied has reached a steady-state, and that in the former period there was an increasing rate of mRNA synthesis. These inferences may not be completely justified for the marrow cell synthesis of stroma, since the mechanism of biosynthesis of this cell constituent is not yet understood. Nevertheless, the inhibition of induced stroma synthesis by actinomycin suggests the involvement of mRNA synthesis in that process, while the inhibition by puromycin indicates the need for protein (enzyme) synthesis. Furthermore, Krantz and Goldwasser¹ have shown that erythropoietin causes an early increase in the synthesis of an RNA fraction that has some of the properties of mRNA.

The data so far accumulated do not offer a choice between two alternative explanations of the linear increase of labeled product with time due to erythropoietin. Either all the sensitive cells respond to the hormone at the same time and incorporate glucosamine at a constant rate as long as erythropoietin is present, or there is a constant rate of formation of sensitive cells, with time, from the pool of potentially erythropoietin-inducible marrow cells. In the latter case, the response during the entire incubation period may be thought of as the sum of a number of short bursts of incorporation by the induced fraction of the marrow cell population. Recent reports based on studies of synchronous cultures of bacteria¹² and yeast¹³ suggest that enzyme synthesis occurs in bursts during particular phases of the cell cycle. Our observation that stimulated glucosamine incorporation continues for several hours after erythropoietin is removed from the culture medium, and subsequently proceeds at the control rate is suggestive of a mechanism involving a burst of activity by a fraction of the cells. If the hormone were not removed from the medium these bursts would add up to the type of incorporation curve seen previously.^{11,12}

The inhibitory effect of actinomycin added 15 hours after erythropoietin is not seen for another 3.5 hours (Figure 3). If the inhibitor has an immediate effect on mRNA synthesis this lag would indicate an average lifetime of 3 to 4 hours for those mRNA's that are involved in synthesis of the

enzymes responsible for erythropoietin-stimulated glucosamine incorporation.

The fact that colchicine has no effect on stimulated glucosamine incorporation for periods up to 23 hours would suggest that there is no mitotic activity after differentiation has been induced by erythropoietin. However, erythropoietin-stimulated hemoglobin synthesis is materially diminished by colchicine, indicating that some cell division must occur in the non-inhibited cultures.² These apparently contradictory data might be reconciled by assuming that in a fixed time a constant amount of glucosamine is incorporated per unit of hormone. When induced cells are permitted to divide, this amount is distributed among them and their progeny. When mitosis does not occur, the cells still take up glucosamine but it is distributed among a smaller number of cells. When (and if) it becomes possible to work with cultures of relatively homogenous cells this hypothesis may be tested by autoradiographic methods.

It is worth noting that the data in Table 3 show that actinomycin completely inhibits erythropoietin action when it is present at a ratio of $0.25 \mu\text{g}/10^6$ cells. Figure 2 shows that, under the same conditions of incubation, but where actinomycin is present at a ratio of $0.156 \mu\text{g}/10^6$ cells there is only 79 per cent inhibition at the 24-hour period while in the first few hours there is a very slight inhibition. These observations suggest that at least two processes are being inhibited by actinomycin, the one being more sensitive to the inhibitor than the other. The less sensitive reaction may involve the ultimate formation of the enzymes concerned with the biosynthesis of stroma, while the more sensitive reaction may lead to the production of cells that can be induced by erythropoietin. This hypothesis would explain our observations, since the smaller amount of inhibitor would then stop production of inducible cells while permitting those already present to be induced and show stimulated glucosamine incorporation. The larger amount of inhibitor would stop both processes. This type of model is formally analogous to that proposed by Lajtha and Oliver.¹⁴ Further tests of this concept are obviously in order.

ACKNOWLEDGMENTS

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ON THE MECHANISM OF ERYTHROPOIETIN-INDUCED DIFFERENTIATION
IV. SOME CHARACTERISTICS OF ERYTHROPOIETIN ACTION
ON HEMOGLOBIN SYNTHESIS IN MARROW
CELL CULTURE*

By

S. B. Krantz[†] and E. Goldwasser

In previous papers^{1,2} we have described a marrow culture system that responds to erythropoietin with a marked increase in hemoglobin production. Further work with this system has shown that erythropoietin may act by way of the stimulation of DNA-dependent RNA synthesis.^{3,4} Since the hormone is believed to act on the primitive stem cells of the marrow, inducing the formation of newly differentiated erythroblasts,⁵⁻⁸ study of its molecular mechanism of action should prove useful in understanding the chemical events leading to differentiation. In this paper we will describe some of the characteristics of erythropoietin action on the cells of rat bone marrow which constitute the target tissue for the hormone.

MATERIALS AND METHODS

The marrow cells from rats fasted 3 days were cultured, and the extent of hemoglobin synthesis was evaluated, as previously described.¹ For some of the experiments smaller dishes (35 x 10 mm) or tubes (16 x 150 mm) were used for the incubation of the cells.[‡] All incubations were done in the presence of 5 per cent CO₂ and 95 per cent air at 37°.

Crude sheep plasma erythropoietin concentrates, generously supplied by the United States Public Health Service Study Section on Hematology, were further purified to yield 2 fractions which had potencies of approximately 260 and 950 units/mg of protein.[§]

Rats were made polycythemic by intraperitoneal injection of washed rat red cells daily until there were no detectable reticulocytes in the circulation.

RESULTS

The effect of increasing erythropoietin levels on the 8-h incorporation of ⁵⁹Fe into heme at different concentrations of cells is shown in Figure 1. The same data are plotted differently in Figure 2 to show the effect of increasing cell number at constant hormone levels.

A series of experiments was carried out to determine the time required for effective contact of the cells with erythropoietin, the rate of the effective attachment, and the amount of hormone either utilized or destroyed by the cells during the incubation. For these experiments the cells were first incubated in closed plastic culture tubes in a horizontal position for the times indicated. The cells were then collected by centrifugation at 250 x g for 2 min and the superna-

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† Postdoctoral Fellow of the U. S. Public Health Service.

‡ The plastic dishes and tubes were bought from Falcon Plastics, Inc.

§ E. Goldwasser and C. Kung, unpublished information.

Figure 1. Log-dose response curves with various concentrations of cells. Cultures incubated in 60 x 15 mm dishes with total volume of 3.0 ml. ⁵⁹Fe was added after 24 h of incubation, cultures stopped at 32 h. Erythropoietin used had potency of 950 units/mg of protein. Numbers on each curve indicate the number of nucleated cells/mm.³ Each point is the mean of duplicates.

Figure 2. Effect of cell number on response to erythropoietin. (Same data as in Figure 1.) Each curve is labeled with units of hormone per ml.

tant medium was replaced by medium to which no erythropoietin had been added but which had been incubated with the same number of cells for the same times. This procedure was necessary in order to eliminate any possible effect of "conditioned medium"⁹ during the total incubation time. After 24 hours of incubation in the 2 stages, ⁵⁹Fe bound to rat plasma was added to the cultures and incubation continued for 23 hours longer, at which time the amount of label in heme was determined by acid butanone extraction.

To allow for any effects of manipulation, the controls for this type of experiment consisted of cultures incubated in tubes, and centrifuged at the same times as the experimental tubes but resuspended without change of medium. Subsequent manipulations were the same for both sets of cultures. The results, shown in Figure 3, are expressed in terms of the control cultures (100 per cent) that were exposed to the action of erythropoietin for the total incubation time (upper horizontal line). The mean value for those samples that had no added erythropoietin is shown by

Figure 3. Effect of time of contact with erythropoietin on hemoglobin synthesis. Cultures contained $15 \cdot 10^6$ nucleated cells/ml in a total volume of 0.8 ml and 0.1 units/ml of erythropoietin (260 units/mg of protein). Cultures were stopped at 47 h. Upper horizontal line represents the effect of the hormone present for the total incubation time (100%), lower horizontal line represents control cultures with no added erythropoietin. Shaded area shows the range of values for this control (4 experiments with triplicate samples). Vertical bars on the curve show standard deviations of the mean values, except for the points at 14 and 16 h, which were done only in duplicate, and show the range.

the lower horizontal line. The data demonstrate that cells in contact with the hormone for periods of up to about 4 hours show no subsequent increase in hemoglobin synthesis. After this time there is a steady increase in effectiveness of the hormone which reaches half maximum at about 14-15 hours.

Because this early lag in the action of the hormone may have been due to the conditions of transfer of the marrow cells from the animal to the situation in vitro (as is the case with some animal cells^{10,11}), a "transfer" experiment, similar to the one described above was done after 9 hours of preincubation in the absence of erythropoietin. The results shown in Figure 4 indicate that part if not all of the lag period may be ascribed to the changed environment of the cells,

Figure 4. Effect of time of contact with erythropoietin on hemoglobin synthesis after 9 h of preincubation. Cultures contained $11 \cdot 10^6$ nucleated cells/ml in a total volume of 0.8 ml and 0.1 units/ml of erythropoietin (260 units/mg of protein). Hormone was added 9 h after starting, cells were transferred at 21 h, ^{59}Fe added at the same time and cultures stopped at 48 h. Vertical bars indicate range of triplicate samples.

since the preincubation period eliminates or greatly shortens the contact time necessary to elicit a response.

In order to determine how much erythropoietin was utilized by the cells in the experiment summarized in Figure 3, the medium removed at each interval was, in turn, assayed for hormone content by incubating it with other cells. Cells from cultures of the same age, incubated with medium containing no added erythropoietin, were collected by centrifugation and were incubated further with the medium from the "transferred" cultures. At each time interval, other replicate cultures in hormone-free medium were given 0.1 units of erythropoietin. The data in Fig-

ure 5 are again expressed as per cent of total response in these latter samples, and show that the amount of hormone utilized or destroyed by the marrow cells in culture is too small to be detected by this method. The continual decrease in responsiveness with time will be discussed below.

Figure 5. Assay of erythropoietin remaining in supernatant medium. Conditions as in Figure 3. Open circles represent incubated medium derived from cells, filled circles represent newly added erythropoietin at same concentration (0.1 units/ml). Cells were placed in dishes (35 x 10 mm) at 14 h, ^{59}Fe added at 24 h and cultures stopped at 47 h. Each point represents the mean of 9 samples except those for 14 and 15 h which had 3 and 6 samples respectively. Vertical bars indicate standard deviations of the means.

Evidence of a short or absent lag period can be obtained from other types of experiments using marrow cells from rats made artificially polycythemic by injections of washed red cells. Since the endogenous level of hormone in these animals should be lower than in either normal or fasted rats, the sensitivity of their marrow cells to exogenous erythropoietin should be increased.⁵ The data in Table 1 show that there is a demonstrable stimulation of hemoglobin synthesis in cultures of such marrow cells within 4 hours. A similar effect can also be seen if the cultures are incubated in a medium containing fetal calf serum instead of rat plasma as the main protein component.³

Since a very small fraction of the available erythropoietin is utilized by the time when the hormone effect on hemoglobin synthesis has reached half its maximum, the decline in hemoglobin synthesis after 35 hours must be due to factors other than depletion of the hormone. It may be due to the exhaustion of a nutrient essential for stimulated hemoglobin synthesis but not for cell division, and for this reason we attempted to replenish a portion of the medium every 24 hours. The cells settle to the bottom of the plates sufficiently well to permit removal of about 85 per cent of

Table 1

EARLY EFFECT OF ERYTHROPOIETIN ON
HEME SYNTHESIS BY MARROW CELLS
FROM POLYCYTHEMIC RATS

Cultures started with $13 \cdot 10^6$ nucleated cells/ml from polycythemic rats, total volume 0.9 ml; ^{59}Fe and erythropoietin added at 0 h and incubation stopped at 4 h. Erythropoietin fraction had 260 units/mg protein. Values given are means of triplicate samples with range indicated in parentheses.

Sample	m μ moles of heme
Control	0.081 (0.075-0.092)
Erythropoietin (0.1 unit)	0.101 (0.100-0.102)

the supernatant fluid and its replacement by fresh medium. In this experiment, freshly drawn rat serum was used in place of rat plasma, and erythropoietin was added to the new medium just before its addition to the cells. The results, shown in Figure 6, indicate that change of medium had no effect on the controls without erythropoietin, which declined steadily. In the cultures with added erythropoietin, however, hemoglobin synthesis was maintained for an additional 24 hours and subsequently fell off to a low level. By 76 hours the marrow cell cultures were extensively overgrown by a monolayer of fibroblasts, and hemoglobin synthesis had ceased.

Figure 6. Effect of medium replacement of erythropoietin stimulation of hemoglobin synthesis. Cultures were started with $11 \cdot 10^6$ nucleated cells/ml in dishes (35 x 10 mm) with total volume of 0.8 ml. Controls (open circles) had no added erythropoietin, experimental (filled circles) had 0.1 units of hormone per ml (260 units/mg). Arrows indicate times when 86% of the medium was replaced in both control and experimental (solid lines). Solid lines represent cultures in which medium was not replaced. Each point is the mean of duplicates and indicates the middle of the 4-h incubation period with ^{59}Fe .

DISCUSSION

Although the data presented in this paper are in terms of heme synthesis, it is now known that virtually all of the heme radioactivity found in marrow cell culture is, in fact, derived from hemoglobin.² This finding justifies the use of the simple measurements of heme radioactivity as an indication of hemoglobin synthesis which we use as one indication of differentiation toward erythroid function.

The log dose-response curves seen in Figure 1 are consistent with those seen in vivo for erythropoietin¹² and in general for many hormones.¹³ The effect of increasing cell number, however, was unexpected (Figure 2). A simple model involving the interaction of a definite number of hormone molecules with susceptible cells would predict a linear relationship between rate of hemoglobin formation and cell number rather than the sigmoid curves found. Since the amount of hormone in the supernatant medium was not detectably depleted by the cells during incubation times of up to 15 hours (Figure 5), the shape of the lower section of curve cannot be due to any phenomenon related to cooperative interactions between the cells and erythropoietin. The most likely explanation would seem, at this time, to be that a particular aggregate of a definite number of cells can synthesize hemoglobin at a rate that is greater per cell than either single cells or aggregates containing a different number of cells. Although we take pains to prepare suspensions of isolated cells, we do find evidence of aggregation during the course of incubation. The shape of the upper end of the curve could be due to one or more of several types of saturation phenomena. Quite obviously, this is a subject that requires further intensive investigation.

The progressive decrease in responsiveness of the cells with time of incubation seen in Figure 5 is consistent with our earlier observations that the rate of hemoglobin synthesis in vitro declines steadily until it is negligible in both control and stimulated cells. In the latter, the decline starts after the wave of marked increase in rate of synthesis due to the hormone was terminated. This loss of function of marrow cells is not due to a loss of erythropoietin since the hormone is present essentially at the original concentration throughout the incubation period. It may be due, rather, to the exhaustion of some required nutrient. This suggestion is supported by the data showing some prolongation of hemoglobin synthesis when the medium is renewed (Figure 6).

Whether a lag time is inherent in erythropoietin action on marrow cells in vitro is still not completely settled. If such a lag time does exist it must be a rather short one in spite of the apparent 6 hour delay in response to erythropoietin seen in Figure 3. There are several lines of evidence that support this contention. The data in Table 1 indicate that, with marrow from polycythemic rats, increased hemoglobin synthesis due to erythropoietin is evident after only 4 hours of incubation. We have previously shown that erythropoietin can effect an increase in hemoglobin synthesis in marrow from rats fasted for 3 days in the first 4 hours of incubation,³ and that the hormone can stimulate RNA synthesis within the first 15 min of incubation.⁴ The apparent lag time in the data in Figure 3 may actually be an increase in hemoglobin synthesis that is too small to be measured under these conditions. The expedient of preincubating, however, seems to make the system more responsive to the hormone, so that the effect of as little as 4 hours of contact time can be easily seen (Figure 4).

From these data and from work previously described¹⁻⁴ a tentative model for the mechanism of erythropoietin action may be proposed. The hormone acts on a primitive cell (stem cell) to induce differentiation into erythroid cells⁵⁻⁸ by initiating the formation of one or more species of

messenger RNA, which determines the pattern of synthesis of specific proteins (such as hemoglobin) that are characteristic of those cells. The data presented in this paper show that there is a continuous action on the cells and that a very small amount of erythropoietin is needed for "induction."

It is possible that the continuous action of erythropoietin on hemoglobin synthesis is exerted solely within those target cells initially stimulated. This may be visualized as a cumulative increase of messenger RNA within a cell as time of contact with the hormone progresses. It is unlikely however that it is a complete explanation, in view of the continually decreasing amount of cell RNA as erythroid maturation progresses.^{14,15} An interpretation more consonant with our knowledge would be that additional cells are being stimulated by the hormone as time progresses and that the rate of increase in hemoglobin synthesis is the resultant of the rate of generation of susceptible cells, the different rates of hemoglobin synthesis in the various types of erythroid cells, and the rate of loss of capacity to synthesize hemoglobin. Additional evidence for this view is presented in the accompanying paper.¹⁵ The continuous action of the hormone as demonstrated in vitro has, indeed, been predicted by the model suggested by Lajtha, Oliver and Gurney⁶ in which the target cells for erythropoietin are randomly in cell cycle and are sensitive to the hormone only at a particular phase. This supposition was made to explain the fact that large doses of the hormone in vivo did not deplete the stem cell pool. This protective feature may be present in vitro as well.

The increase in the rate of hemoglobin synthesis with time of contact with the hormone could represent a cumulative increase in messenger RNA within newly stimulated target cells. The curvilinear increase in heme synthesis might be due to repeated use of the same messenger RNAs in the synthesis of more enzymes and repeated use of these enzymes to make more hemoglobin. This is consistent with our observations on the relatively long lifetime of the messenger RNAs involved in hemoglobin synthesis.³

It is only too clear that most of the questions concerning the mechanism of erythroid differentiation still remain to be answered. Some of the data presented in this paper on the use of marrow cells in vitro for the study of erythropoietin action may be useful for further investigation into this subject.

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THE HEMOLYSIS OF RED CELLS FROM PATIENTS WITH PAROXYSMAL
NOCTURNAL HEMOGLOBINURIA BY PARTIALLY PURIFIED
SUBCOMPONENTS OF THE THIRD COMPLEMENT
COMPONENT*

By
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The role of the C[‡] system in the hemolysis of PNHE has been emphasized recently by the demonstration in this laboratory that PNHE lysis and classical C' dependent immune lysis had similar cation requirements and pH optima.^{1,2} In addition, the enhancing effects of C'1a, C'1 esterase and C'1 activators upon PNHE hemolysis suggested that the early steps of C' activation ultimately leading to PNHE lysis occurred in the fluid phase rather than in apposition to the cell membrane.³ Implicit in the latter speculation was the concept that late acting C' components were capable of direct attack upon PNHE.

The present report demonstrates that PNHE can be hemolyzed directly by partially purified subcomponents of the C'3 complex. Central to this thesis is the observation that PNHE exposed to C'3a are converted to an intermediate complex (designated PNHEC'_{3a}), which is susceptible to hemolysis by dilute human serum in the presence of Na₃HEDTA. In addition, the serum C' components involved in the hemolysis of PNHEC'_{3a} are found to be identical with those involved in the lysis of EAC'_{1,4,2,3a}.

MATERIALS AND METHODS

The following have all been described previously: the preparation of barbital buffered saline (BBS) at various pH's;² the preparation of Na₂MgEDTA-BBS containing 1.5 or 2.6 x 10⁻² M Na₂MgEDTA;^{2,4} the collection of guinea pig and human blood and the isolation and storage of their respective sera;^{2,3} the preparation and storage of sera deficient in the various components of C' and RP;³ the preparation of sensitized sheep red cells both with and without the use of Na₃HEDTA-BBS;² the preparation of EAhuC'_{1,4,2} and EAgpC'_{1,4,2};² the collection and storage of NHE and PNHE;³ the adjustment of serum pH;³ the preparation of serum containing EDTA salts;³ the estimation of in vitro acid hemolysis;³ the assay for C'1 esterase activity.³

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‡ The following abbreviations are used: C', complement; C'1, C'2, C'3, C'4, the first, second, third or fourth component of complement; R1, R2, R3, R4, RP, serum lacking the designated complement component, or properdin; C'1a, C'2a, activated first or second complement components; C'1q, C'1r, C'1s, subcomponents of the first component of complement; E, erythrocytes; A, antibody or amboceptor; EA, sensitized erythrocytes (usually sheep red cells); EAC'...., sensitized erythrocytes bearing complement components as designated by subscript; PNHE, paroxysmal nocturnal hemoglobinuria; PNHE, red cells from patients with paroxysmal nocturnal hemoglobinuria; NHE, normal human red cells; hu, human; gp, guinea pig; EDTA, ethylenediamine tetraacetate.

The subcomponents of human C'3 are designated by the notation C'3a, C'3b, C'3c (Cf. Taylor and Leon,⁷).

Preparation of C'3a by TEAE chromatography. C'3a was prepared as outlined by Müller-Eberhard.⁵ C'3a obtained by the initial chromatographic isolation was useful in preparing PNHEC'_{3a}, but most of the experiments were performed with C'3a that had been further purified by rechromatography. Following rechromatography, the C'3a-containing fractions were pooled, dialyzed against 0.02 ionic strength phosphate buffer, pH 5.4, and the precipitate redissolved in 5 to 10 ml of phosphate buffered (pH 7.0) isotonic saline. These fractions deteriorated slowly, and by the end of 6 to 8 weeks their activity had often disappeared. The activity could be preserved by storing the fractions at -20° C in 50 per cent glycerol, and dialyzing them against 0.15 M NaCl before use.

Formation of the PNHEC'_{3a} complex. Nine different batches of C'3a were prepared, of which 6 were rechromatographed; no C'1 esterase activity was detectable in any of these preparations. All were effective in converting PNHE to PNHEC'_{3a}. After both reactants had been prewarmed to 37°, an aliquot of C'3a was added to a cell button of PNHE, and the mixture was incubated at 37° for 5 minutes. Volumes of PNHE ranged from 0.01 to 0.5 ml, and volumes of C'3a ranged from 0.1 to 2 ml. The mixture was then placed on ice and washed twice (2° C) with 10 ml 0.15 M NaCl. In certain experiments 1 ml of human serum, pH 6.5, containing 0.015 M Na₃HEDTA was added directly to the PNHEC'_{3a} cell button, and hemolysis was carried out at 37° for 30 minutes. When the PNHEC'_{3a} complex was prepared in bulk, the washed cells were suspended to a concentration of 20 to 40 per cent and 0.05 ml was added to tubes containing 1-ml aliquots of the Na₃HEDTA human serum or other reagents. The dose of PNHEC'_{3a} employed in each hemolytic test ranged from an equivalent of 0.01 to 0.02 ml packed cells, and the 100 per cent hemolysis O. D. 540 mμ (determined by freeze and thaw) ranged from 1.5 to 4.0.

Isolation of the serum factors involved in PNHEC'_{3a} hemolysis. Four hundred fifty to six hundred ml of human serum was mixed with one-tenth volume of 0.1 M Na₃HEDTA, pH 7.4, and dialyzed against 12 liters 0.02 ionic strength acetate buffer, pH 5.4, containing 10⁻³ M Na₃HEDTA for 16 to 24 hours. The euglobulin precipitate was collected by centrifugation, washed twice in dialysis buffer, and dissolved in 25 ml phosphate buffer, ionic strength 0.15, pH 7.4, containing 1 per cent NaCl, and 10⁻³ M Na₃HEDTA. After removal of insoluble material and lipid by low and high speed centrifugation,⁵ the protein solution was dialyzed overnight against starting buffer. Starting buffer was either 0.15 ionic strength phosphate, pH 7.4 (2 experiments) or 0.09 ionic strength phosphate buffer, pH 7.4 (2 experiments) both containing 10⁻³ M Na₃HEDTA. No significant differences were noted when the elution patterns for these two buffers were compared. The dialyzed protein solution was cleared of any precipitate by low speed centrifugation, and was placed on a 2 x 40 cm column of DEAE resin previously equilibrated with starting buffer. The columns were developed using a linear ionic strength gradient (starting buffer as described; limit buffer, 0.5 M NaCl in starting buffer). In 3 experiments, 1000 ml of each buffer was employed for gradient development. In a single experiment, in order to avoid trace contamination of the C'3 subcomponents by each other, 2000 ml of each buffer was employed. Twenty ml fractions were collected at a flow rate of 15 to 30 ml/hour. The individual fractions were dialyzed overnight against 0.135 M NaCl containing 10⁻³ Na₃HEDTA. The entire isolation procedure was carried out at 4° C. After dialysis, the fractions were analyzed for C'1q, C'1r, and C'1s activities,⁴ for C'3a activity,^{4,6} for lytic activity against PNHEC'_{3a}, and for hemolytic activity against EAC'_{1,4,2,3a}. Comparable results were obtained in all four experiments. Activities isolated by this technique were stored at -85° C.

Methods of Assay

C'1q, C'1r, C'1s. The procedure was similar to that outlined by Lepow and his associates.⁴

C'3a. This activity was usually measured using EAgpC'_{1,4,2}. A portion of the fraction to be tested was added to a 5×10^8 cell button of EAgpC'_{1,4,2} together with 4 ml of a 1:5000 dilution of guinea pig C' in Na₃HEDTA-BBS, pH 7.4.⁴ Hemolysis was carried out at 37° for 60 minutes. Appropriate blanks and controls were included. The activity of C'3a was also measured with EAhuC'_{1,4,2}, using a 1:800 or 1:1000 dilution of human serum in Na₃HEDTA-BBS. Similar results were obtained.

Serum factors involved in PNHEC'_{3a} hemolysis. All tests for the ability of DEAE chromatography fractions to hemolyze PNHEC'_{3a} were carried out in the presence of 7.5×10^{-3} M Na₃HEDTA with Na₃HEDTA-BBS, pH 6.5, as diluent where indicated. Assays for these activities were carried out as follows: 0.1 ml of the fraction to be tested was added to 1 ml Na₃HEDTA-BBS, pH 6.5, together with 0.1 ml of a fraction of known activity, and 0.05 ml of a 20 per cent suspension of PNHEC'_{3a}. Hemolysis was carried out by incubating the mixture at 37° for 30 minutes. Appropriate controls employing one or other of the test fractions with PNHEC'_{3a}, and both test fractions with PNHE, were included.

Serum factors involved in the lysis of EAC'_{1,4,2,3a}. These assays were carried out using EAC'_{1,4,2} and C'3a; identical results were obtained with EAgpC'_{1,4,2} and EAhuC'_{1,4,2}. Hemolysis could be produced by a combination of EAC'_{1,4,2}, C'3a, an early DEAE fraction (C'3b) and a late DEAE fraction (C'3c).⁷ The fractions to be tested were diluted 1:25 or 1:50 in Na₃HEDTA-BBS, pH 7.4. A 5×10^8 button of EAC'_{1,4,2} at 0° C was combined with an aliquot of C'3a, 2 ml of a dilution of known activity, and 2 ml of a dilution of the fraction to be tested. The cell suspension was then incubated at 37° for 60 minutes. Appropriate controls (with and without C'3a) and blanks were always included.

Other Procedures

Immunoelectrophoresis was performed on rechromatographed C'3a preparations, using a rabbit antihuman serum,^{*} rabbit antihuman gamma globulin serum,^{*} and rabbit antihuman β_{1c} -globulin serum. The latter was prepared from an antihuman C' serum[†] containing antibodies to both β_{1e} -globulin and β_{1c} -globulin by absorption with human red cells coated with incomplete cold antibody.⁸

Coombs tests were performed on both PNHE and NHE with and without exposure to C'3a. Two-hundredths ml PNHE or NHE were exposed to 0.3 ml saline or C'3a containing 5000 μ g protein/ml for 5 minutes at 37°. They were then washed 4 times with 10 ml saline at 2° C and suspended to a 3 per cent cell concentration. One drop of each cell suspension was added to 2 drops of antiserum dilution in 10 x 75 cm test tubes. After incubation at 37° for 30 minutes with frequent agitation, the cell suspensions were allowed to stand at room temperature for 60 minutes and the hemagglutination pattern was noted. A smooth carpet of cells was taken as a 4+ pattern, and a dense central button as a negative pattern. All antisera were heated at 56° for 30 minutes and absorbed 3 times at 0° with one-half volume of washed human red cells prior to use.

^{*} Prepared by Behringwerke Ag. and distributed in the United States by Lloyd Bros. Inc., Cincinnati, Ohio, 45229.

[†] Prepared by Centr. Lab. Bloedtr. Dienst, Amsterdam, Holland and kindly supplied by Dr. Fred Rosen, Childrens Medical Center, Boston, Massachusetts.

In certain experiments antiserum was absorbed with C'3a prior to use; 1 volume of antiserum, 1 volume of C'3a (5000 μ g protein/ml), and 8 volumes of saline were mixed and incubated at 37° for 30 minutes.

Preparation of the TEAE* and DEAE† resins for column chromatography was as described by Müller-Eberhard, Nilsson, and Aronsson⁵ and by Lepow and his associates.⁴ Conductivity measurements were made with a conductivity bridge.‡ pH measurements were made with a Beckman Zeromatic pH meter. A Zeiss PMQ II spectrophotometer and cuvettes with a 1-cm light path were employed in all measurements of optical density. Protein determinations were made by a modified Folin technique⁹ with human gamma globulin as the standard. The absorbancy at 280 $m\mu$ of a 1 per cent protein solution of partially purified C'3a was 12.

All the experiments were performed on more than one occasion, with different cells and different reagent preparations, and the results were always reproducible.

RESULTS

Figure 1 shows the relationship between C'3a activity as measured by EAC'_{1,4,2} lysis in a 1:5000 dilution of guinea pig serum, and the material isolated by TEAE chromatography that was capable of transforming PNHE to PNHEC'_{3a}. Only the fraction pools containing C'3a activity as measured by EAC'_{1,4,2} were capable of PNHEC'_{3a} formation. On one occasion the peak fractions containing C'3a activity from the initial chromatographic separation were dialyzed against 0.15 M NaCl and used directly in an unsuccessful attempt to convert PNHE to PNHEC'_{3a}.

PNHEC'_{3a} formation: cation requirements, pH optima, kinetics, etc. Twenty-five to 50 μ g C'3a protein/ml effects a barely discernible conversion of PNHE to PNHEC'_{3a} (Figure 2). With concentrations of C'3a above 500 μ g protein/ml the slope of PNHEC'_{3a} formation diminishes.

Experiments to determine the cation requirements revealed that the intermediate complex was formed equally well in the presence of Na₂MgEDTA and Na₃HEDTA as in the presence of Ca⁺⁺ and Mg⁺⁺. It was concluded, therefore, that neither of these divalent cations was necessary for PNHEC'_{3a} formation, and that fluid phase C'1 or C'2, even if present as contaminants in our C'3a preparation, were not participating in the conversion.¹⁰ Nevertheless, the possibility that a cell bound complement was playing a role in C'3a attachment was not excluded. Accordingly, PNHE were decayed at 37° for 2 hours in BBS or in Na₃HEDTA-BBS, and subsequently were tested for their ability to form PNHEC'_{3a}. The results revealed that such decay did not affect the subsequent formation of PNHEC'_{3a}, and eliminated the possibility that C'1 or C'2 need be on the cell for PNHEC'_{3a} formation to occur.^{11,12}

PNHEC'_{3a} formation was completely inhibited by low temperature (0° C). Furthermore, preheating C'3a at 56° for 30 minutes completely destroyed its ability to effect PNHEC'_{3a} formation. During exposure of C'3a to 56° much of the protein formed a white precipitate. PNHEC'_{3a} forma-

* Two different TEAE resins were employed with comparable results: 1) Cellex-T, Biorad Laboratories, Richmond, Calif., Lot no. B-1548, capacity 0.76 mEq/gm. 2) Gallard-Schlesinger Chem. Mfg. Corp., Garden City, N. Y., Lot no. A 3849, capacity 0.73 mEq/gm.

† Two different DEAE resins were employed with comparable results: 1) Selectacel, Schleicher and Schuell, Keene, New Hampshire, capacity 0.95 mEq/gm. 2) Gallard-Schlesinger Chem. Mfg. Corp., Garden City, N. Y., Lot no. A 4769, capacity 1.18 mEq/gm.

‡ Model RC16B2, Industrial Instruments, Inc., Cedar Grove, New Jersey.

Figure 1. Isolation of C'3a by TEAE chromatography. The bracketed fractions were pooled, the protein was reprecipitated and dissolved in a small volume of phosphate buffered saline. The pooled fractions were tested for C'3a activity using EAC'1,4,2 and PNHE.

tion proceeded equally well at pH's 6.5, 7.0 and 7.5; slight inhibition was noted at pH 6.0.

The kinetics of PNHEC'3a formation superficially resembled those of the formation of EAC'1,4,2^{10a, 13}. The complex formed rapidly, reached an optimum at approximately 5 minutes of PNHE exposure to C'3a, and then began to decline in activity (Figure 3). The decay of PNHEC'3a could be impeded by low temperature (Figure 4). The rate of decay at 37° was much slower than that associated with EAC'1,4,2 and did not follow first order kinetics. In addition, PNHEC'3a maintained at 37° in the presence of a large excess of C'3a were able to escape decay. The optimum time of exposure of PNHE to C'3a for PNHEC'3a formation varied with the concentration of C'3a and also differed slightly with various batches of C'3a; however, a 5 minute exposure of PNHE to C'3a at 37° yielded satisfactory results in all instances. It should be noted that no hemolysis of PNHE occurred during prolonged exposure to C'3a, or during subsequent decay at 37°; thus C'3a was unable, by itself, to produce PNHE lysis.

Various studies on the lysis of PNHEC'3a: pH optimum, effects of serum dilution, etc. The PNHEC'3a complex is identifiable by its ability to hemolyze in the presence of Na₃HEDTA. Red cells from 10 patients with PNH were studied during the course of these experiments; none was capable of significant hemolysis in Na₃HEDTA-containing human serum. All were capable of

Figure 2. Effect of C'3a protein concentration on PNHEC'3a formation. Two-hundredths ml PNHE was mixed with 0.5 ml C'3a at various protein concentrations. After 5 minutes incubation at 37° C, the cells were washed and 1 ml of a 1:25 dilution of human serum in Na₃HEDTA-BBS, pH 6.5, was added to the cell button.

Figure 3. Kinetics of PNHEC'3a formation. Two-tenths ml PNHE was mixed with 5 ml of C'3a (500 μg protein/ml) at 37°. At the designated time intervals 0.5 ml aliquots were removed and added to cold 0.15 M NaCl. After washing, the cell buttons were hemolyzed in 1 ml undiluted human serum, pH 6.5, containing 0.015 M Na₃HEDTA.

Figure 4. Effect of temperature upon the decay of PNHEC'_{3a}. A 20% suspension of PNHEC'_{3a} was maintained at the designated temperatures; 0.05 ml aliquots were removed at intervals and tested for hemolysis in 1 ml of a 1:16 dilution of human serum in Na₃HEDTA-BBS, pH 6.5.

conversion to the intermediate complex PNHEC'_{3a}, as measured by susceptibility to hemolysis in the same reagent. Hemolysis of PNHEC'_{3a} in Na₃HEDTA human serum could proceed over an expanded pH range, with a broad optimum occurring between pH 6.5 and 6.0 (3 experiments).

The native PNH cell loses its capacity to undergo hemolysis in very low dilutions of serum, and is usually resistant to hemolysis in serum dilutions of 1:4 or higher.¹ In marked contrast, the PNHEC'_{3a} complex was susceptible to hemolysis by extremely high dilutions of human serum in Na₃HEDTA-BBS; substantial hemolysis occurred in dilutions of human serum as high as 1:1024 (Figure 5). Peak hemolysis was usually observed between serum dilutions of 1:4 and 1:32: Most tests for PNHEC'_{3a} hemolysis utilizing whole serum were therefore performed in a 1:16 or 1:25 dilution of serum in Na₃HEDTA-BBS, pH 6.5.

The hemolysis of PNHE in whole human serum is dependent upon the presence of all four major C' components as well as properdin.³ Figure 6 shows the major C' components that are necessary to support hemolysis of PNHEC'_{3a}. Serum heated at 56° for 30 minutes would no longer hemolyze PNHEC'_{3a}. PNHEC'_{3a} were no more susceptible to acid hemolysis in native human serum than PNHE. Hemolysis of PNHEC'_{3a} proceeded satisfactorily in R1, R2, R4, and RP. The only R reagent totally and consistently lacking in ability to hemolyze PNHEC'_{3a} was R3, suggesting that some part of the C'3 complex was responsible for the lysis of PNHEC'_{3a}.

Isolation of the serum factors involved in PNHEC'_{3a} hemolysis: their identity with C'3b and C'3c. Figure 7 demonstrates the results obtained when the DEAE resin chromatographic fractions were analyzed for various hemolytic activities. No single fraction was capable of hemolyzing either PNHEC'_{3a} or EAC'_{1,4,2,3a}; the combination of an early fraction (C'3b) with a late fraction (C'3c) was effective in lysing both intermediate complexes. The pattern of elution of C'3b and C'3c as

Figure 5. Ability of PNHEC'_{3a} to hemolyze in various dilutions of human serum. Aliquots of a single PNHEC'_{3a} preparation were added to 1 ml of serial two-fold dilutions of human serum in Na₃HEDTA-BBS, pH 6.5. The undiluted serum contained 0.015 M Na₃HEDTA.

measured by PNHEC'_{3a} resembled closely that measured by use of EAC'_{1,4,2,3a}. Table 1 illustrates the need for both C'3b and C'3c in the lysis of all the C'3a intermediate complexes studied.

The effect of C'3a on normal human erythrocytes. Two of the undiluted rechromatographed C'3a preparations were effective in forming an intermediate complex NHEC'_{3a} which is suscep-

Figure 6. Ability of various R reagents to support the hemolysis of PNHEC'_{3a}. All hemolytic tests were carried out in 1 ml portions of the indicated reagent containing 0.1 ml 0.15 M Na₃HEDTA at pH 6.5. PNHE will not hemolyze in serum containing Na₃HEDTA nor in any R reagent containing Na₃HEDTA (not shown).

Figure 7. Isolation of the serum factors (C'3b, C'3c) involved in PNHEC'3a hemolysis by DEAE chromatography. The arrow indicates the start of the NaCl gradient. Starting buffer 0.09 ionic strength phosphate, pH 7.4; 2000 ml each starting and limit buffers were employed for gradient development.

tible to hemolysis in dilute Na_3HEDTA human serum. Table 2 illustrates this effect and compares the efficacy of this transformation with that effected simultaneously against PNHE. The PNHE used were approximately 40 times more susceptible to complex formation than were NHE. Another C'3a preparation was even more effective in transforming NHE to NHEC'3a, but unfortunately decayed before PNHE could be obtained for parallel assay. It should be emphasized that all of the experiments cited herein concerning the formation of PNHEC'3a were done with amounts of, or preparations, of C'3a that were ineffective in forming NHEC'3a. Table 3 shows the capacity of PNHE from 6 patients to combine with C'3a and contrasts their capacity to do so with NHE and with red cells from 3 patients displaying substantial reticulocytosis not related to a hemolytic anemia. The ability of PNHE to react with C'3a is not shared by other red cell populations containing a high proportion of reticulocytes, and is not attributable to a special affinity of young red cells for C'3a.

Immunoelectrophoresis of rechromatographed C'3a. Two precipitin bands were identifiable by immunoelectrophoresis. One of them, β_{1c} -globulin, showed a minor component with greater anodal electrophoretic mobility. The other band detected by rabbit antihuman serum was not further identified. No 7S γ globulin was present in the C'3a preparations (Figure 8).

Coombs tests. Following exposure to C'3a both PNHE and NHE gave a positive hemagglutina-

Table 1

ROLE OF C'3b AND C'3c IN THE LYSIS OF PNHEC'_{3a}, EAhuC'_{1,4,2,3a} AND EAgpC'_{1,4,2,3a}

A [*] Reagent	Lysis of		B [†] Reagent	Lysis of		C [‡] Reagent	Lysis of	
	PNHEC' _{3a} O.D. 540 mμ	PNHE O.D. 540 mμ		EAhuC' _{1,4,2} + C'3a O.D. 540 mμ	EAhuC' _{1,4,2} O.D. 540 mμ		EAgpC' _{1,4,2} + C'3a O.D. 540 mμ	EAgpC' _{1,4,2} O.D. 540 mμ
C'3b	0.002		C'3b	0.016	0.022	C'3b	0.007	0.008
C'3b + C'3c	0.535	0.040	C'3b + C'3c	0.372	0.019	C'3b + C'3c	0.329	0.011
C'3c	0.028		C'3c	0.013	0.018	C'3c	0.016	0.021
1:25 hu serum (Na ₃ HEDTA-BBS)	0.360	0.023	1:1000 hu serum (Na ₃ HEDTA-BBS)	0.145	0.016	1:5000 gp serum (Na ₃ HEDTA-BBS)	0.671	0.057
Na ₃ HEDTA-BBS blank	0.010	0.020	1:20 hu serum (Na ₃ HEDTA-BBS)		0.410	1:100 gp serum (Na ₃ HEDTA-BBS)		0.955
100%	2.100	1.870	Na ₃ HEDTA-BBS blank	0.021		Na ₃ HEDTA-BBS blank	0.021	0.059
			100%	1.280	1.280	100%	1.010	1.010

^{*} C'3b + C'3c from DEAE isolation no. 1.[†] C'3b + C'3c from DEAE isolation no. 4, diluted 1:25 in Na₃HEDTA-BBS, pH 7.4. C'3a isolated by TEAE chromatography (batch 11/27).[‡] C'3b + C'3c from DEAE isolation no. 3, diluted 1:50 in Na₃HEDTA-BBS, pH 7.4. C'3a isolated by TEAE chromatography (batch 10/27).

Table 2
COMPARISON OF PNHE (case Pe) AND NHE REACTION WITH C'3a

C'3a concentration	NHE		PNHE	
	O.D. 540 m μ	Lysis*	O.D. 540 m μ	Lysis*
μ g protein/ml		%		%
5000 [†]	0.144	7.8	0.970	59.5
1000 [‡]	0.010	0.5	0.465	28.5
500 [‡]	0.002	0.1	0.404	24.8
250 [‡]	0.002	0.1	0.185	11.3
125 [‡]	0.000	0.1	0.104	6.4
Saline control	0.004	0.2	0.007	0.4
100% (F and T)	1.840		1.630	

* In 1:25 hu serum, Na₃HEDTA-BBS, pH 6.5.

[†] Two-tenths ml C'3a/0.01 ml NHE or PNHE, 37°, 5 minutes.

[‡] Five-tenths ml C'3a/0.01 ml NHE or PNHE, 37°, 5 minutes.

Figure 8. Immunoelectrophoresis of rechromatographed C'3a. Anti-human serum: antiserum to whole human serum; anti- γ -globulin: antiserum to human γ -globulin; anti- β_{1c} : antiserum to β_{1c} -globulin.

Table 3

ABILITY OF RED CELLS FROM VARIOUS INDIVIDUALS TO FORM AN INTERMEDIATE COMPLEX WITH C'3a*

Patient	Diagnosis	Treatment	Reticu- locytes	Concentration of C'3a (μg protein/ml)										100% lysis O.D. 540 mμ
				2250 [†]		450 [†]		225 [†]		113 [†]		0		
				O.D. 540 mμ	Lysis [§]	O.D. 540 mμ	Lysis [§]	O.D. 540 mμ	Lysis [§]	O.D. 540 mμ	Lysis [§]	Saline control		
												O.D. 540 mμ	Lysis [§]	
Case 4	PNH		% N.D. [§]	0.865	47.3	0.331	18.1	0.220	12.1	0.115	6.3	0.020	1.1	1.824
Case 3	PNH		N.D.	0.298	15.7	0.129	6.8	0.112	5.9	0.037	2.0	0.008	0.5	1.890
Case 8	PNH		N.D.	0.303	15.4	0.120	6.1	0.076	3.9	0.027	1.4	0	0	1.965
Case 2	PNH		N.D.	0.840	48.2	0.293	16.8	0.237	13.6	0.075	4.3	0	0	1.745
Case 5	PNH		N.D.	0.541	32.6	0.272	16.4	0.185	11.1	0.084	5.1	0.030	1.8	1.660
Case Pa	PNH		N.D.	0.357	15.5	0.142	6.2	0.105	4.6	0.038	1.7	0	0	2.30
Ro	P.A.	B ₁₂ 5 days	26.8	0	0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0	0	1.546
Ho	Folic acid deficiency	Folic acid 5 days	15.5	0.015	0.8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.018	1	1.900
Mi	Folic acid deficiency	Folic acid 5 days	41.9	0.008	0.4	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.008	0.4	1.940
Ya	Normal		N.D.	0	0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0	0	2.45

* The C'3a used was a rechromatographed glycerolized portion, dialyzed prior to use in 0.15 M NaCl. The original batch, tested immediately after isolation, was unable to form an intermediate complex with normal red cells at a concentration of 6750 μ g protein/ml. All the data shown in this table were obtained during a single experiment.

[†] Five hundredths ml 20% suspension of cells + 0.2 ml C'3a, 5 minutes, 37°.

[‡] Five-hundredths ml 20% suspension of cells + 0.5 ml C'3a, 5 minutes, 37°.

[§] One ml 1:25 dilution of human serum in Na₃HEDTA-BBS, pH 6.5.

[§] N.D. = Not done.

^{||} "Native" PNHE. No transfused cells.

tion reaction with antihuman serum; cells similarly treated did not react with anti- β_{1c} -globulin or anti 7S γ -globulin. Preabsorption of the antihuman serum with C'3a abolished the positive hemagglutination reaction (Table 4).

Table 4

HEMAGGLUTINATION PATTERN OF HUMAN RED CELLS EXPOSED TO C'3a
WHEN TESTED WITH A RABBIT ANTIHUMAN SERUM

Cell type	Antiserum	Antiserum dilution						
		1:10	1:20	1:40	1:80	1:160	1:320	Saline control
PNHE	Antihuman serum	0	0	0	1+	0	0	0
NHE	Antihuman serum	0	0	0	0	0	0	0
PNHE exposed to C'3a	Antihuman serum	4+	4+	3+	2+	1+	±	0
NHE exposed to C'3a	Antihuman serum	4+	3+	2+	±	0	0	0
PNHE exposed to C'3a	Antihuman serum absorbed with C'3a	0	0	0	0	0	0	0
NHE exposed to C'3a	Antihuman serum absorbed with C'3a	0	0	0	0	0	0	0

PNHE and NHE exposed to C'3a gave negative patterns when tested with anti- γ -globulin serum and anti- β_{1c} -globulin serum. See METHODS for details of procedure.

DISCUSSION

Although much has been learned about the mechanism of C' action during the preceding decade,¹⁴ the precise nature of the damage inflicted upon the red cell membrane that leads to subsequent lysis is not known, nor is it known which of the several C' components acts to produce the ultimate membrane injury. Certain C' components presumably function only to activate later-acting C' components necessary for the development of progressive stages in immune lysis. Since the C' components thus involved need not be present on the cell membrane when lysis actually occurs, they are probably not involved in membrane injury. C'1, for example, is dispensable after the attachment of C'4 and C'2, and cells in the state of EAC'_{4,2} can be readily lysed by C'3.¹² Similarly C'2 is only necessary for the attachment of C'3a; cells in the state EAC'_{1,4,3a} (or presumably EAC'_{4,3a}) need only be exposed to C'3b and C'3c for lysis to occur.^{15*} Since no method is yet known of removing or inactivating cell bound C'4, the possibility that it plays a role in the terminal phases of immune lysis cannot be excluded with certainty. It is, however, equally possible that C'4 is not required beyond the activation and attachment of C'2 and that cells in the state

* Linscott and Nishioka (Ref. 15) have identified four subcomponents of guinea pig C'3 and have called them C'3a, C'3b, C'3c, and C'3d. Although definitive data on the relationship between the human C'3 subcomponents described in this report and guinea pig C'3 subcomponents are not presently available, for the purpose of discussion the following have been assumed:

hu C'3a $\approx$ gp C'3c + gp C'3b
hu C'3b $\approx$ gp C'3a
hu C'3c $\approx$ gp C'3d

EAC'_{3a} would hemolyze in the presence of C'3b and C'3c. Such a possibility is suggested by the experiments described in this paper.

The ability of PNHE to combine directly with partially purified C'3a and to form an intermediate complex susceptible to lysis by C'3b and C'3c confirms the observations of Rosen made with radioiodinated C'3a,¹⁶ and substantiates our earlier speculations that PNHE might be susceptible to lysis by late-acting complement components attaching directly from the fluid phase.³ Taken together with the findings of Jenkins that PNHE remaining intact after acid hemolysis in whole serum bear on their surface C'3a, but not C'4,¹⁷ the present observations permit us to make certain approximate statements about the mechanism of PNHE lysis by acidified whole human serum in vitro. There remains little reason to doubt that in vitro lysis of PNHE is effected by the C' system, and not by any special or esoteric species of serum factors, inhibitors, or activators.¹⁸ In this respect, PNHE lysis does not differ from classical immune hemolysis. A striking difference between these two C' dependent hemolytic systems does exist, however, in the localization of the earlier stages of the C' sequence. In immune lysis these early stages, mediated by the antibody coat, occur in apposition to the cell membrane; in PNHE lysis they take place in the fluid phase. The susceptibility of the PNHE hemolytic system to dilution implies that the fluid phase activation steps involved are not very efficient; and the lack of significant C' consumption during PNHE lysis confirms this impression.¹⁹ In addition, PNHE, lacking a C' activating mechanism (antibody coat), are deprived of the ability to exert any direct influence on the initiation and localization of C' action.^{3,20} The need for participation of fluid phase early C' components in PNHE lysis in acid serum is attested to by the dependence of acid hemolysis on Mg^{++} ,¹ implying that at least C'2 is involved in fluid phase events. Taken in the framework of our current knowledge of the role of C'2 in C' dependent lysis, it seems reasonable to conclude that these C'2 mediated fluid phase events are preceded by action of C'1²¹ (and possibly C'4), and that they are concerned with rendering C'3a capable of attachment to PNHE (? activation).^{3,22,23} One can "drive" the entire sequence of early phase events and increase resultant PNHE lysis by adding to the system C'1 activators, C'1a, or C'1 esterase.³ That a delicate balance exists in this fluid phase mechanism is shown by the inhibition of PNHE lysis by an excess of these "driving" materials.³ This inhibition is probably due to inactivation of C'2 (?C'4) and/or C'3a. Bypassing the early fluid phase events concerned with C'3a activation, and placing C'3a directly on PNHE, erases all distinction between the PNHEC'_{3a} complex and its counterpart in immune hemolysis, EAC'_{1,4,2,3a}. Both cells are now susceptible to lysis in the absence of Ca^{++} or Mg^{++} , both can be hemolyzed by high dilutions of human serum, both will hemolyze in R1, R2, and R4 but not R3,⁷ and both can be attacked directly and hemolyzed by purified C'3b and C'3c.⁷ While all of these statements can be made with some degree of certainty in terms of in vitro PNHE lysis, their exact relationship in vivo PNHE hemolysis remains less clear. It does not, however, seem unreasonable to assume that the C' system plays a similar role in intravascular PNHE destruction. Recognition of this fact may help in the search for effective ways of controlling the rate of red cell destruction in vivo.

Müller-Eberhard has shown that serum exposed to zymosan at 15° C suffers conversion of its β_{1c} -globulin to β_{1a} -globulin, a hemolytically inert component.⁶ Such serum treatment is the method by which RP serum is ordinarily prepared.²⁴ We have previously demonstrated that RP serum can be made to hemolyze PNHE by the addition of a potent C'1 activator.³ The present report demonstrates that RP serum is effective in hemolyzing PNHEC'_{3a}. These observations, taken together point to a close relationship between C'3a and properdin.

When subjected to immunoelectrophoretic analysis, our C'3a preparations display at least two distinct protein fractions. One of them, β_{1c} -globulin, shows a minor subcomponent with greater anodal electrophoretic mobility; this could represent β_{1a} -globulin, an inactive fragment of β_{1c} -globulin formed by aging,⁵ or β_{1g} -globulin, the reaction product of C'2a and β_{1c} -globulin.²² The second constituent has not been identified but may be either β_{1f} -globulin or C'6, which have been shown by Müller-Eberhard to be present in C'3a fractions prepared by his original method.²³ The hemagglutination studies demonstrate the presence of a serum protein constituent on PNHEC'3a which is distinct from β_{1c} -globulin. Whether this serum protein fraction is β_{1f} -globulin, C'6, or both has not been ascertained.

We have previously cited evidence that eliminates the possibility that PNHE are in the state PNHEC'1,4,2.³ Since the C'3a utilized during the present studies contains at least two and probably three C'3a subcomponents,²³ it might be argued that one of these activities is already attached to PNHE and effects the attachment of the others. However, the observations of Linscott and Nishiooka,¹⁵ and of Nilsson and Müller-Eberhard²³ indicate that the components comprising C'3a activity require cell bound C'2a for attachment during immune lysis. The experiments utilizing decayed PNHE clearly demonstrate that cell bound C'2 plays no direct role during the attachment of C'3a. While PNHE presumably go through a state equivalent to PNHEC'3a in vivo, such cells are probably destroyed by C'3b and C'3c almost immediately, since PNHE studied in vitro are resistant to hemolysis in the absence of Mg^{++} .¹

Certain activators of C'1 such as polyinosinic acid and dextran sulfate are capable of initiating lysis of NHE as well as PNHE.³ The mechanism of induction of such hemolysis has been presumed to involve the same fluid phase C' component events as those involved in PNHE hemolysis. Viewed in the light of these prior observations, it is not surprising to discover that NHE are also susceptible to direct attack by C'3a, C'3b, and C'3c, albeit to a far lesser extent than PNHE. The demonstration that both diseased and normal human red cells are capable, under proper conditions, of combining directly with C'3a without the direct mediation of an antibody coat or earlier cell bound C' components, suggests that human red cells bear on their surface receptor sites for such an attachment. The PNH red cell differs from NHE in having a greater number of such sites, or in having them more accessible to fluid phase C'3a. Thus, whatever the nature or pathogenesis of the membrane lesion of PNHE (and it is clear that the present report throws little light on this fundamental question) we can at least say that it increases the affinity of the PNHE membrane for C'3a when compared with the normal human red cell membrane, and that this affinity makes PNHE susceptible to attack by fluid phase processes of C' activation ordinarily insufficient to attack NHE. If the biochemical nature of the membrane site involved in C'3a attachment were to be established it might provide an avenue through which more definitive understanding of the nature of the membrane lesion in PNH could be obtained.

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OBSERVATIONS ON THE ROLE OF PROPERDIN, 0° FACTOR, POLYINOSINIC
ACID, AND THROMBIN IN THE HEMOLYSIS OF PAROXYSMAL
NOCTURNAL HEMOGLOBINURIA ERYTHROCYTES*

By

S. Yachnin†

Absorption of serum at 15° C with zymosan renders the serum deficient in properdin and destroys the capacity of such RP serum to hemolyze paroxysmal nocturnal hemoglobinuria erythrocytes (PNHE).¹ Absorption of serum with zymosan at 0° C does not remove properdin, but does remove a zymosan specific fraction ("0° factor"), so that upon incubation with fresh zymosan at 15° C properdin is no longer affected.² Recent work in this laboratory has defined the PNHE hemolytic system as one dependent upon fluid phase complement (C') activation resulting in red cell membrane destruction as a result of direct attack by C'3 subcomponents.³ C'1 esterase or C'1 activators have been shown to enhance PNHE lysis, and in addition certain C'1 activators such as polyinosinic acid (poly I) may initiate the hemolysis of normal human erythrocytes (NHE), presumably by similar fluid phase complement reactions.⁴ Thrombin has also been shown to enhance PNHE lysis but the mechanism of this enhancement has been the subject of considerable controversy. The observations shown in Table 1 were designed to assess the relative role of properdin and "0° factor" in native and poly I enhanced PNHE lysis, and to compare the stimulating effect of poly I upon PNHE hemolysis with the ability of thrombin, ‡ both absorbed with NHE, and unabsorbed, to enhance PNHE lysis.

Removal of "0° factor" from serum does not affect the ability of serum to support PNHE hemolysis under any of the experimental conditions cited. Removal of properdin, on the other hand, does inhibit the capacity of serum to support PNHE hemolysis at both pH 7.6 and 6.5. The ability to hemolyze PNHE can be restored to RP serum by the addition of poly I; the latter stimulates PNHE hemolysis in RP serum much more effectively at pH 6.5 than at pH 7.6, but at neither pH does poly I-enhanced hemolysis proceed as well as in normal serum. Absorption of thrombin with NHE diminishes greatly its ability to stimulate PNHE cell lysis. Addition of NHE absorbed thrombin to normal serum at pH 6.5 does not result in significant enhancement of PNHE hemolysis. The latter observation agrees with that of Hinz,⁵ but is at variance with the experience of two other groups^{6,7} who have found residual capacity for PNHE acid hemolysis stimulation in thrombin that has been exhaustively absorbed by NHE. More in keeping with these latter reports however, we have found that absorbed thrombin can provoke moderate enhancement of PNHE hemolysis in normal serum at pH 7.6, and can partially restore hemolytic capacity to RP serum at pH 6.5. Removal of properdin from serum diminishes, but does not completely abolish, the ability of poly I to provoke hemolysis of NHE at pH 6.5.⁴

Properdin and zymosan-specific 0° factor are not required for poly I-induced hemolysis,

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‡ Thrombin, bovine, Upjohn Co., Kalamazoo, Mich.

Table 1

EFFECT OF PROPERDIN AND "0° FACTOR" DEPLETION UPON POLY I ENHANCED
HEMOLYSIS OF PNH RED CELLS; COMPARISON OF POLY I
EFFECT WITH THROMBIN EFFECT

The hemolytic test system consisted of 1 ml of the appropriate serum, acidified where indicated with 0.3 N HCl, 0.05 ml of a 20% suspension of PNHE in 0.15 NaCl, and 0.1 ml of 0.15 NaCl or 0.1 ml of the appropriate material dissolved in 0.15 NaCl, added in the order mentioned. After incubation at 37° for 30 minutes under oil, the tubes were centrifuged and read at 540 m μ in a Zeiss PMQ II spectrophotometer against the appropriate serum blank.

Addition to test serum	Percentage hemolysis					
	pH 7.6			pH 6.5		
	Normal serum	0° supernatant serum	RP serum	Normal serum	0° supernatant serum	RP serum
Saline (0.1 ml)	14	14	1	51	54	2
Poly I - 1 μ mole P	58	56	15	81	81	64
Thrombin - 50 U	66	65	63	73	73	68
Absorbed thrombin - 50 U	27	24	1	53	53	28

although poly I-induced hemolysis is reduced somewhat in RP serum. 0° factor is also not required for native PNHE hemolysis. Viewing properdin as related intimately to the recognized components of C',⁸ and recognizing the peculiar affinity of zymosan for C'3,⁹ one could speculate that properdin is in fact a subcomponent of C'3. Such a possibility has been reinforced by our recent observations that the intermediate complex, PNHE β_{1c} formed by exposure of PNHE to purified human β_{1c} globulin (C'3a), is highly susceptible to hemolysis in RP serum in the presence of Na₃HEDTA.³ Bearing in mind the concepts recently developed on the special relationship of late-acting C' components to PNHE hemolysis,^{3,4} it is conceivable that partial depletion of such a C'3 subcomponent might critically affect a hemolytic system dependent upon threshold amounts of activated fluid phase, late-acting C' components (native PNH hemolysis); might partially, but not completely, inhibit a hemolytic system capable of activating more efficiently residual fluid phase late-acting C' components (poly I-induced hemolysis); and might inhibit only slightly, if at all, a hemolytic system capable of activating and utilizing the residue of such a factor with great efficiency at the cell membrane (classical immune hemolysis).

Most observers agree that much of the capacity of commercial bovine thrombin to enhance PNHE hemolysis is attributable to the presence of red cell hetero-antibodies.⁵⁻⁷ Our observations, however, in agreement with those of Jenkins and associates,⁶ and Crosby and Benjamin,⁷ demonstrate that even after extensive absorption with human red cells, such bovine thrombin is still capable, under certain conditions, of enhancing PNHE hemolysis. Jenkins and associates have clearly shown that thrombin per se is not involved in this enhancement; since the factor in question is heat stable they conclude that it is nonabsorbable antibody.⁶ On the basis of the mechanisms discussed in this communication, however, this enhancing factor could be either a substance that, like poly I, is capable of activating C'1, or the bovine counterpart of one of the heat stable C'3 subcomponents of guinea pig serum (C'3c, C'3b).¹⁰ That at least one of the bo-

vine C'3 subcomponents can function in a heterospecific C' system has been demonstrated by Linscott and Nishioka who used bovine plasma as a rich source of C'3d.¹⁰

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PSEUDOURIDINE FORMATION: EVIDENCE FOR RNA AS AN INTERMEDIATE*

By

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Since the description of 5-ribosyluracil (pseudouridine) as a component of cellular RNA's,^{1†} several investigators have attempted to elucidate the mechanism by which this nucleoside is formed. Robbins and co-workers, using doubly labeled uridine and 5-fluorouridine, concluded that both the ribose and pyrimidine moieties of uridine are direct precursors for ψ uridine formation in yeast.² Based on these findings, the same authors suggested an intramolecular rearrangement of the uridine base and sugar, but their data were insufficient to decide whether this rearrangement occurred before or after uridine incorporation into RNA.

On the other hand, several investigators have suggested that ψ uridine might be formed by a 3,5-diribosyluracil intermediate³ or by a direct condensation of uracil with ribose-5-phosphate to form ψ UMP.⁴ The enzyme ψ UMP synthetase which catalyzes this condensation reaction has been partially purified from extracts of *Tetrahymena pyriformis*.⁴ Although these findings indicate that ψ uridine may be formed before nucleotide polymerization, it is still possible that ψ uridine, found in the framework of cytoplasmic RNA's, is formed after polynucleotide assembly. We now report evidence that in *Escherichia coli*, ψ uridine formation requires the prior synthesis of RNA. Agents that inhibit ribonucleotide polymerization also prevent ψ uridine formation.

The following experiments were conducted with either intact or lysed spheroplasts from *E. coli*. Intact spheroplasts were prepared as previously reported.⁵ Lysed cells were prepared by sedimenting the spheroplasts, and suspending them in a hypotonic medium containing 0.02M tris, pH 7.5, 0.002M MgCl₂ and 0.014M 2-mercaptoethanol. The labeled precursors used were uracil-2-¹⁴C,[§] UTP-¹⁴C, or uridine-labeled cRNA-¹⁴C (complementary RNA). UTP-¹⁴C was prepared by deamination of uniformly labeled CMP[¶] according to the procedure of Lohman,⁶ purification of the UMP product by chromatography on Dowex-1 (formate), and conversion to UTP by a yeast extract containing uridylate kinase activity.⁷ The labeled UTP was not purified from the kinase mixture but used as such after it was heated at 90°C for 3 minutes. Paper electrophoresis of this mixture indicated that more than 95 per cent of the radioactivity migrated as UTP, the remainder being distributed between UMP and UDP. The above procedure was used to avoid the presence of labeled ψ uridine nucleotides found in commercial preparations of uridine-¹⁴C nucleotides. This difficulty was only partially overcome since our preparation of

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‡ The following abbreviations are used: RNA and DNA for ribo- and deoxyribonucleic acid; ψ uridine, ψ UMP and ψ UTP for 5-ribosyluracil and the mono- and triphosphate nucleotides of 5-ribosyluracil; CMP, UMP, UDP, CTP, UTP, ATP, and GTP as the mono-, di- and triphosphate nucleotides of cytidine, uridine, adenosine, and guanosine; PEP, phosphoenolpyruvate; and cRNA for the synthetic RNA prepared with RNA polymerase.

§ Schwarz Bioresearch.

¶ New England Nuclear Corporation.

UTP-¹⁴C still contained some contaminant (approximately 0.05 per cent) which behaved like ψ uridine in the assay procedure and contributed to the high baselines found. The contaminant did not appear to be ψ UTP-¹⁴C since synthetic cRNA-¹⁴C, prepared with the same labeled UTP, contained little radioactive ψ uridine. Radioactive cRNA was prepared with the highly purified RNA polymerase from Micrococcus lysodeikticus, E. coli DNA being used as template and UTP-¹⁴C as the only labeled substrate.⁷ The synthetic cRNA was freed from UTP-¹⁴C of the polymerase reaction mixture by repeated precipitation with ethanol and exhaustive dialysis against 0.1M NaCl and then against water. Pseudouridine used as carrier was isolated from human urine by the procedure of Adler and Gutman⁸ as modified by Goldberg and Rabinowitz.⁹

The procedure routinely used for isolating ψ uridine was as follows: (1) The entire reaction mixture was hydrolyzed with alkali, treated with alkaline phosphatase and the total labeled nucleosides were isolated by adsorption onto Dowex-1 (acetate) and eluted with acetic acid (see footnote to Table 1); (2) Carrier uridine and ψ uridine were added to the isolated nucleoside fraction which was then concentrated and subjected to descending paper chromatography in a mixture of butanol and water (86:14), (3) Pseudouridine was eluted and passed over a column (1 by 7 cm) of Dowex-50 (H⁺) in 0.05N HCl; the acid-eluate was neutralized and the labeled nucleoside was concentrated by treatment with Dowex-1 (acetate) as described above; (4) The concentrate was subjected to paper chromatography in the butanol-water system a second time; (5) The ψ uridine band was eluted and subjected to paper chromatography in a system containing isopropanol, acetic acid and water (6:3:1). After the second butanol-water chromatography, the radioactivity in the ψ uridine region remained fairly constant, with little change on subsequent chromatography and little radioactivity detectable in uridine. In some experiments further discrimination between the uridine isomers was made by the action of yeast uridine nucleosidase,¹⁰ which specifically cleaves the glycosidic linkage of uridine but does not attack ψ uridine.¹¹

Intact E. coli spheroplasts readily incorporate uracil-¹⁴C into an acid-insoluble form and this incorporation is quite sensitive to the presence of actinomycin D. Analysis of the total spheroplast radioactivity (acid-soluble and insoluble material) revealed that ψ uridine labeling was far less extensive in actinomycin-treated cells than in cells that had not been exposed to the antibiotic (Table 1). Because of the inhibitory effect exerted by actinomycin D on the DNA-directed synthesis of RNA¹² the results of this experiment suggested the possible involvement of RNA in the conversion of uridine to ψ uridine.

Osmotically lysed spheroplasts are highly viscous, and actively catalyze ribonucleotide polymerization. Since RNA synthesis with lysed E. coli preparations is dependent upon fairly intact endogenous DNA, ψ uridine formation should also be dependent upon intact DNA if RNA is involved in the conversion mechanism. Table 2 illustrates that lysed preparations of E. coli spheroplasts actively incorporate UTP-¹⁴C into an acid-precipitable form and that this incorporation is inhibited by the presence of deoxyribonuclease, or by the omission of a full complement of ribonucleoside triphosphates. Analysis of the total labeled uridine material in these reaction mixtures indicated that a significant increase in ψ uridine labeling, as compared to the zero time control, occurred only when UTP-¹⁴C incorporation into RNA was not impaired. In a similar experiment with labeled UTP and lysed spheroplasts, actinomycin D also inhibited the formation of labeled ψ uridine.

The experiments described above provide indirect evidence that RNA may be an interme-

Table 1

EFFECT OF ACTINOMYCIN D ON URACIL-¹⁴C INCORPORATION INTO
PSEUDOURIDINE WITH ESCHERICHIA COLI SPHEROPLASTS

The incubation mixture, final volume 10.9 ml contained 10 ml of spheroplasts in PAM medium⁶ (approximately 1×10^9 cells/ml) and 0.532 μ moles of uracil-2-¹⁴C (64.9 $\times 10^6$ counts/ μ mole). In addition, one incubation mixture contained 250 μ g of actinomycin D. After 2 hours of incubation at 37°C, the cells were sedimented by centrifugation, suspended in 2 ml of 0.4 N KOH and held at 37°C for 18 hours. The hydrolysate was acidified with 70 per cent HClO₄, iced for 1 hour, centrifuged, and the supernatant exposed to the action of intestinal alkaline phosphatase (1 mg/ml) overnight at 37°C after adjustment to pH 9 in a final volume of 10 ml. The reaction was stopped by heating for 10 minutes in a boiling water bath, cooled, filtered, taken up to a volume of 100 ml and adjusted to pH 10.5 with NH₄OH. The ¹⁴C-nucleosides were concentrated by adsorption onto a 6 cm² column of Dowex-1 (acetate), elution with 0.1N acetic acid and evaporation to dryness under vacuum. The labeled residue was dissolved in a minimum volume of water, carrier uridine and ψ uridine were added and the ψ uridine reisolated by the chromatographic and Dowex-50 (H⁺) treatment described in the text.

Expt. no.	Labeled precursor	Actinomycin D	Total acid-insoluble counts/min	Total counts/min in ψ uridine
1	uracil-2- ¹⁴ C	+	0.18×10^6	775
2	uracil-2- ¹⁴ C	-	1.54×10^6	4160

Table 2

DEPENDENCE OF ψ URIDINE LABELING ON DNA AND
RIBONUCLEOSIDE TRIPHOSPHATES

The complete mixture (2.0 ml) contained 200 μ M of tris, pH 7.5; 4 μ moles each of ATP, GTP, and CTP; 0.075 μ moles of uniformly labeled UTP-¹⁴C (approximately 150 mc/mM); 8 μ moles of phosphoenolpyruvate; 80 μ g of pyruvate kinase; 6 μ moles of MnCl₂; 4 μ moles of spermidine and osmotically lysed spheroplasts (approximately 1.5 to 2.0×10^{11} cells). The mixtures were incubated at 30°C for 30 minutes, stopped with 0.20 ml of 4N KOH, held at 37°C for 18 hours and then ψ uridine was isolated as described in Table 1.

Expt. no.	Basic mixture	Labeled substrate	Acid-insoluble counts	Counts in pseudouridine
1	Complete (zero)	UTP- ¹⁴ C	0.05×10^6	4,985
2	Complete	UTP- ¹⁴ C	1.33×10^6	17,380
3	Complete + DNase (50 μ g)	UTP- ¹⁴ C	0.11×10^6	4,820
4	Omit ATP, CTP, GTP and PEP	UTP- ¹⁴ C	0.31×10^6	6,740

Table 3

FORMATION OF ψ URIDINE- ^{14}C FROM URIDINE-LABELED cRNA- ^{14}C

The complete system (2.0 ml) was similar to that described in Table 2 except that UTP- ^{14}C was omitted and *E. coli* cRNA- ^{14}C served as the substrate. The reaction mixtures were incubated at 30° C and stopped with 5 per cent acid. The precipitate was washed twice with 5 per cent acid, once with ethanol-ether (3:1) and analyzed for the presence of labeled ψ uridine. In Expt. 2 (but not Expt. 1), the ψ uridine counts were subjected to the action of yeast uridine nucleosidase and then re-chromatographed in the butanol- H_2O system.

Expt. no.	Experimental conditions			Total counts/min			
	Complete system	DNase (100 μg)	Actinomycin D (30 μg)	cRNA- ^{14}C added	cRNA- ^{14}C recovered	Uridine	Pseudouridine
1	Zero	+	-	6.5×10^6	5.8×10^6	-	336
	Incubated (10 minutes)	+	-	11.1×10^6	6.6×10^6	-	1280
2	Zero	-	+	10.6×10^6	9.5×10^6	74	110
	Incubated (15 minutes)	-	+	17.8×10^6	10.1×10^6	39	670

diates in the conversion of uridine to ψ uridine and that the conversion takes place after polynucleotide synthesis. A more direct proof of this conclusion can be provided by using uridine-labeled cRNA- ^{14}C . With RNA as a substrate, ψ uridine formation should be independent of agents which specifically block RNA synthesis. Table 3 shows that when uridine-labeled cRNA- ^{14}C is reisolated after incubation with a lysed spheroplast preparation in the presence of either deoxyribonuclease or actinomycin D, it now contains an increased amount of labeled ψ uridine as compared to the unincubated controls. The low ψ uridine radioactivity cannot be attributed to contamination by labeled uridine since the latter contained negligible counts (experiment 2). Nearly twice as much cRNA- ^{14}C was added to the "incubated" mixtures as to the "zero" time controls to compensate for the vigorous ribonuclease activity exhibited by the lysed bacterial preparations. Since ψ uridine analysis was performed only on the cRNA- ^{14}C recovered by acid-precipitation, and since in both experiments the amount recovered from the control and experimental mixtures was nearly similar, the excess cRNA- ^{14}C that was degraded to acid-soluble material should not have altered the results, assuming that the liberated uridine- ^{14}C nucleotides were not reincorporated into RNA. The presence of deoxyribonuclease or actinomycin D in the incubated mixtures should have prevented this recycling process. Although the total ψ uridine radioactivity was relatively small compared to the RNA counts analyzed, the results are consistent with those of the other experiments presented here. Furthermore, if one assumes that most of the cellular RNA- ψ uridine is primarily located in transfer RNA, that in the RNA polymerase transcription process only 0.02 per cent of the synthetic cRNA represents transfer RNA, and that 15 to 20 per cent of the uridine in transfer RNA represents ψ uridine, then the ψ uridine counts given in Table 3 are within the correct order of magnitude for the amount of labeled cRNA used. The rather small amount of labeled ψ uridine formed in these experiments also suggests that the enzyme(s) responsible for this reaction must be highly specific in the selection of uridine residues in the polynucleotide chain for conversion to ψ uridine.

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ISOLATION AND CHARACTERIZATION OF RIBONUCLEOPROTEIN PARTICLES FROM HEART MUSCLE*

By

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Ribosomes have been prepared from a number of mammalian tissues and used to great profit in the study of protein biosynthesis. Isolation from muscle of ribonucleoprotein particles that catalyze significant incorporation of amino acid into protein has, however, proven difficult. The difficulty may arise in part from characteristics inherent in the tissue. Liver, for example, yields ribosomes that are highly active in the incorporation of amino acid into protein,¹ but liver tissue is differentiated for the production of protein for export. Muscle, on the other hand, consists mainly of structural and contractile proteins, and synthesizes little or no protein for utilization elsewhere in the body. There are in addition technical difficulties. The conventional procedures for the fractionation of homogenates by differential centrifugation and deoxycholate treatment give, in the case of muscle, small yield of ribonucleoprotein particles with low RNA to protein ratios and poor ability to catalyze protein synthesis.² There are recent reports that ribosomes may be isolated from rat skeletal muscle microsomes,³ or from rat cardiac muscle by density gradient centrifugation,⁴ and that the particles so isolated will incorporate amino acid into protein; but the former procedure gives poor yields and the latter requires that the muscle be treated with proteinase, which leads to diminished activity and perhaps to an alteration of the structure of the protein-synthesizing particles.

The present paper presents alternate procedures for the isolation and purification of ribosomes from rat and chick embryo heart muscle; describes the characteristics of the particles; delineates the conditions under which significant incorporation into protein of radioactivity from sRNA charged with ¹⁴C-amino acid occurs; and reports the enhancement of the process by synthetic polyribonucleotide.

Preliminary reports of the work have been published.^{5,6}

MATERIALS AND METHODS

Uniformly labeled L-¹⁴C-phenylalanine (297 mc/mmole) was obtained from New England Nuclear Corporation; nonradioactive amino acids and puromycin hydrochloride from Nutritional Biochemicals Corporation. ATP and GTP were purchased from Sigma Chemical Company; phosphoenolpyruvate and pyruvate kinase from Calbiochemical Company; 5 times recrystallized ribonuclease and 5 times recrystallized chymotrypsin from Worthington Biochemical Company; polyuridylic acid from Miles Chemical Company, and sodium deoxycholate from Fischer Scientific

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Company. The Escherichia coli (strain B) sRNA was obtained from General Biochemicals; it had been stripped of amino acids. Actinomycin C₁ (D) was a gift of Dr. C. Stone, Merck, Sharpe and Dohme Research Laboratories; chloramphenicol was obtained from Searle Laboratories.

Preparation of Ribosomes

Method 1. Male Sprague-Dawley rats, maintained under standard conditions⁷ until they weighed 130 to 160 g, were fasted overnight, killed by decapitation and their hearts removed, blotted free of excess blood, and placed in ice cold medium A (0.05 M Tris-HCl buffer, pH 7.6; 0.01 M MgCl₂; 0.1 M KCl and 0.005 M β -mercaptoethanol). In some experiments hearts from 14 to 18-day-old chick embryos were used. The subsequent preparation in each case was carried out in the cold at 2 to 4°. The tissue was minced, rinsed to remove the remaining blood, and homogenized in 3 vol of medium A in a Waring Blendor at high speed for 2 min (or twice in a Virtis Homogenizer at 25,000 rev/min for 30 sec) and then in a motor driven all-glass Potter-Elvehjem homogenizer for 1 min. The homogenate was centrifuged at 0° for 2 hrs at 105,000 x g (Spinco Model L centrifuge, No. 50 rotor). The supernatant was discarded and the pellet containing all the formed elements of the cell was resuspended in medium B (0.05 M Tris-HCl buffer, pH 7.6; 0.01 M MgCl₂; 0.1 M KCl) and sufficient 2 M sucrose in medium B added to give a final concentration of 0.88 M in four times the original tissue volume. To the suspension was added one-ninth its volume of 2.5 per cent sodium deoxycholate (in 0.05 M Tris-HCl, pH 7.8) so as to give a final concentration of 0.25 per cent. The volumes generally used to suspend the pellet from 25 g of tissue were 46 ml of medium B, 44 ml of 2 M sucrose in medium B, and 10 ml of 2.5 per cent deoxycholate. The suspension was centrifuged at 0° for 30 min at 30,000 x g and the sediment of heavy cellular material discarded; the supernatant was centrifuged again at 30,000 x g for 30 min and the small amount of sediment discarded. The supernatant was finally centrifuged at 0° for 2 hours at 105,000 x g. The ribosomal pellet was rinsed several times with medium B and either stored at -20° or made up in an appropriate volume of suspending medium (0.05 M Tris-HCl buffer, pH 7.5; 0.01 M MgCl₂; 0.1 M KCl; 0.35 M sucrose) and used immediately.

Liver ribosomes were prepared in the same way.

Method 2. The tissue homogenate prepared by Method 1 was centrifuged at 28,500 x g in a Lourdes centrifuge (9RA rotor) for 30 min at 0°; the pellet was discarded. The postmitochondrial supernatant was then centrifuged at 105,000 x g for 60 min to sediment the microsomal fraction. The microsomal pellet was suspended in 4 vol of medium B in which the sucrose concentration was adjusted to 0.88 M. Deoxycholate was then added to a final concentration 0.25 per cent. The suspension was centrifuged for 30 min at 30,000 x g and the sediment was discarded. The ribosomes were then sedimented by centrifugation at 105,000 x g for 60 min.

Preparation of Mitochondria

Hearts from rats were finely minced with a Latapie tissue grinder; hearts from chick embryos with scissors. The minced tissue was homogenized in Medium C (0.02 M EDTA; 0.3 M sucrose; 0.03 M nicotinamide) with 3 strokes of a Potter-Elvehjem glass homogenizer. The homogenate was centrifuged at 600 x g for 10 min at 0° in a Lourdes centrifuge; the supernatant fluid was recentrifuged in the same way. To sediment the mitochondria the supernatant was now centrifuged at 8,000 x g for 10 min at 0°. The mitochondria were washed 3 times with 4 vol of

medium C, being centrifuged after each wash at 8,000 x g for 10 min. Finally, the mitochondria were suspended in 4 vol of 0.25 M sucrose and centrifuged at 600 x g for 10 min; the sediment was discarded.

Before assay of incorporation of amino acid into protein, the mitochondria were treated with ribonuclease to minimize the possibility of contamination by ribosomes. For that purpose the mitochondria were suspended in the following media: 0.02 M Tris-HCl buffer, pH 7.5; 0.05 M KCl; 0.001 M EDTA; 0.001 M AMP; 0.01 M $MgCl_2$; 0.03 M phosphate buffer, pH 7.5; 0.001 M DPN; 0.01 M α -ketoglutarate; 0.02 M nicotinamide.⁸ To each ml of medium 200 μ g of ribonuclease was added and the mixture incubated for 10 min at 37°. After incubation the mitochondrial suspension was cooled to 0° and washed 5 times with medium C (being centrifuged each time at 8,000 x g for 10 min) to remove ribonuclease.

For assay of incorporation of radioactivity into protein the mitochondria were suspended in 1 ml of media that contained: 0.05 M Tris-HCl buffer, pH 7.5; 0.05 M KCl; 0.003 M $MgCl_2$; 0.001 M EDTA; 0.03 M phosphate buffer, pH 7.5; 0.001 M ATP; 0.003 M α -ketoglutarate; 0.5 μ c of ^{14}C -phenylalanine (but no unlabeled phenylalanine) and 30 μ g of each of 19 other ^{12}C -amino acids.

Preparation of ^{14}C -amino acyl-sRNA

Liver amino acyl-sRNA was prepared using sRNA extracted from liver homogenates by the phenol procedure,⁹ and "pH 5 enzyme fraction" prepared from liver by the method of Keller and Zamecnik.¹⁰ The sRNA was charged with L- ^{14}C -phenylalanine and 19 additional ^{12}C -amino acids as described by Liao and Williams-Ashman.¹¹ The E. coli ^{14}C -phenylalanine-sRNA was prepared according to the procedure of Ehrenstein and Lipmann.¹²

To catalyze the transfer of amino acids from charged sRNA to ribosomes, "transfer enzyme" was used; it was prepared by dialyzing the 105,000 x g soluble fraction of either rat liver or rat heart homogenates against 0.01 M Tris-HCl buffer, pH 7.8; 0.01 M $MgCl_2$; 0.01 M KCl; 0.005 M β -mercaptoethanol for 4 hours.

Assay of Incorporation of Radioactivity from ^{14}C -amino acyl-sRNA into Protein

The reaction was carried out in a final volume of 1 ml at 37°. The assay system contained: 50 μ moles Tris-HCl buffer, pH 7.8; 10 μ moles $MgCl_2$; 30 μ moles KCl; 1 μ mole ATP; 1 μ mole GTP; 10 μ moles β -mercaptoethanol; 10 μ moles phosphoenolpyruvate; 10 μ g pyruvate kinase; 1 mg dialyzed 105,000 x g supernatant protein; sRNA-phenylalanine- ^{14}C ; and ribosomes. The quantities of the latter 2 components are given in the tables and figures. The reaction was initiated by the addition of ribosomes and terminated by the addition of 5 ml of ice cold 10 per cent trichloroacetic acid.

Chemical Analyses

The approximate concentration of ribosomes was calculated from the absorbancy at 260 $m\mu$ using an absorbancy coefficient, $E_{1\text{ cm}}^{1\%}$, of 125.¹³ Ribosomal RNA was estimated after alkaline hydrolysis from the absorbancy at 260 $m\mu$ as described by Hecht et al.¹⁴ using an absorbancy coefficient of 32 $mg^{-1} cm^2$, or by the orcinol procedure after extraction with hot 5 per cent trichloroacetic acid;¹⁵ the two methods gave similar results. Ribosomal protein was determined by the Lowry method;¹⁶ "transfer enzyme" (105,000 x g dialyzed supernatant protein) according to Warburg and Christian;¹⁷ and the protein content of the pH 5 fraction by the biuret reaction.¹⁸

For determination of the base ratio, ribosomal RNA was extracted with phenol;¹⁹ hydrolyzed in 0.1 N NaOH at 37° for 18 hours, and the nucleotides separated by ascending paper chromatography²⁰ and assayed from the absorbancy at 260 m μ .

Preparation of Radioactive Protein for Counting

For the assay of incorporation of ¹⁴C-phenylalanine into protein by ribosomes or mitochondria, the protein precipitated by the addition of ice cold 10 per cent trichloroacetic acid was washed free from radioactive amino acid, the RNA and lipids were extracted and the protein dried²¹ and then dissolved in 1.5 ml of formic acid and poured onto tared stainless steel planchets with concentric rings. The formic acid was evaporated, the planchets reweighed and the radioactivity of the protein assayed in a Nuclear-Chicago gas-flow counter with a thin mica window (D-47) having an efficiency of 40 per cent. The standard error of the procedure was less than 3 per cent. The results were corrected for background and for zero time controls; the latter were usually less than 20 counts/min.

RESULTS

Characteristics of Ribonucleoprotein Particles from Heart Muscle

In a series of preliminary and exploratory experiments it was found that ribosomes could be isolated from heart muscle by first preparing microsomes and then treating the microsomes with deoxycholate (method 2), but the yield (50 μ g of ribosomal RNA per g of tissue) was poor. A considerable improvement in the yield (approximately 2.5 fold), and some increase in purity (i.e., higher RNA to protein ratio), was effected when the entire muscle homogenate (105,000 x g pellet) was treated with deoxycholate (method 1). For those reasons, improved yield and greater purity, method 1 was generally used. The ribonucleoprotein particles isolated from rat heart muscle by method 1 had an absorbancy ratio at 260 m μ to that at 280 m μ of 1.5 to 1.7 (similar to that which characterizes nucleoprotein); the absorbancy ratio at 260 m μ to that at 235 m μ varied, with different preparations, between 1.25 and 1.35, indicative of some impurity.¹³ The particles ordinarily contained equal amounts of RNA and protein; the best preparations had in excess of 55 per cent RNA. Analysis of the base composition of the RNA of the particles gave in moles/100 moles; guanine, 35.4; adenine, 20.3; cytidine, 30.2; uridine, 14.1. The base ratio (G + C/A + U) was 1.9; a high guanine plus cytidine content, as was found, is typical of mammalian ribosomal preparations.²² The yield was 0.2 to 0.25 mg of ribosomes or 0.1 to 0.13 mg of ribosomal RNA per g of tissue.

When ribosomes were isolated from heart muscle of 14 to 18-day chick embryos the yield was approximately 5 times greater (670 μ g ribosomal RNA per g of tissue) than could be obtained from heart muscle of young rats. The ribosomes from chick embryo heart usually had a higher concentration of RNA. The improved yield probably reflects a higher ribosomal concentration rather than improved efficiency of isolation. The total RNA content of embryo hearts was 5.2 mg RNA/g of tissue; approximately 5 times greater than that present in young rat hearts (1.1 mg RNA/g of tissue). In each case 10 to 15 per cent of the total cellular RNA was recovered in the ribosomal fraction.

Incorporation of Radioactivity from sRNA-phenylalanine-¹⁴C into Protein by Ribosomes from Rat Heart Muscle—General Characteristics

Ribonucleoprotein particles from heart muscle catalyzed the transfer of radioactivity from

sRNA-phenylalanine- ^{14}C to protein (Table 1) in a reaction that required ribosomes, energy (ATP, phosphoenolpyruvate, pyruvate kinase), GTP, "transfer enzyme," and mono- and divalent cations. Addition of polyuridylic acid caused a 2- to 10-fold stimulation of incorporation of ^{14}C from sRNA-phenylalanine (Table 1; here 5-fold); puromycin depressed incorporation both in the absence (68 per cent) and the presence (70 per cent) of polyuridylic acid (see also Table 3). Significant utilization (13 per cent) of added sRNA-phenylalanine ^{14}C occurred in the basal system and was increased (to 69 per cent) by the addition of polyuridylic acid.

Table 1

INCORPORATION OF RADIOACTIVITY FROM sRNA-PHENYLALANINE- ^{14}C INTO
PROTEIN BY RIBOSOMES FROM RAT HEART MUSCLE:
EFFECT OF POLYURIDYLIC ACID

The reaction was carried out as described in methods. Each tube also contained in 1 ml: 0.5 mg of liver sRNA charged with $7.4 \cdot 10^3$ counts/min of ^{14}C -phenylalanine and 200 μg of ribosomal RNA. Incubation was for 50 min at 37° .

Addition to the medium		Incorporation into protein (counts/min)	Utilization of added radioactivity (%)
Reaction mixture	Polyuridylic acid		
Complete	0	951	13
	+	5020	68
Omit energy *	0	559	8
	+	3005	41
Omit GTP	0	504	7
	+	3470	46
Omit 105,000 x g supernatant	0	514	7
	+	586	8
Complete, add (0.2 μmoles) puromycin	0	312	4
	+	1520	21

* ATP, phosphoenolpyruvate, pyruvate kinase.

Ion Requirements

The requirement for magnesium for incorporation of ^{14}C from sRNA-phenylalanine into protein by rat heart ribosomes shows a sharp optimum at 10 $\mu\text{moles/ml}$ (Figure 1). The optimal concentration of potassium is 100 $\mu\text{moles/ml}$ (Figure 2); ammonium ion can replace potassium. The ion requirements for incorporation of amino acid into protein by ribosomes from heart thus accord with that observed for ribosomes from other tissues.^{23,24}

Effect of Inhibitors

The incorporation of radioactivity from sRNA-phenylalanine- ^{14}C into protein is sensitive to ribonuclease; as little as 0.5 $\mu\text{g/ml}$ of enzymes causes significant (72 per cent) inhibition and inhibition appears near complete at 2 $\mu\text{g/ml}$ (Table 2). Incorporation is also sensitive to inhibition by puromycin but chloramphenicol is without effect at concentrations as high as $1 \cdot 10^{-3}$ M (Table 3).

Figure 1. Effect of magnesium concentration on incorporation into protein of radioactivity from ^{14}C -phenylalanine-sRNA by ribosomes from rat heart muscle. The reaction was carried out as described in methods except for the variation in magnesium concentration. Each tube also contained: 200 μg of polyuridylic acid; 0.5 mg of liver sRNA charged with $2.4 \cdot 10^4$ counts/min of ^{14}C -phenylalanine; and 45 μg of ribosomal RNA prepared by Method 1. The ribosomes had an absorbancy ratio (260/280) of 1.6 and had been washed with solution A from which magnesium and potassium were omitted. Incubation was for 15 min at 37° .

Figure 2. Effect of potassium concentration on incorporation into protein of radioactivity from ^{14}C -phenylalanine-sRNA by ribosomes from rat heart muscle. Conditions were as described for Figure 1 except that the magnesium concentration was 10 mM, and the potassium concentration was varied.

Table 2

EFFECT OF RIBONUCLEASE ON INCORPORATION
INTO PROTEIN OF RADIOACTIVITY FROM ^{14}C -
PHENYLALANINE-sRNA BY RIBOSOMES
FROM RAT HEART MUSCLE

The reaction was carried out as described in methods. Each tube also contained: the concentration of ribonuclease specified; ribosomes prepared by Method 1 (115 μg of RNA); and 0.15 mg of *E. coli* sRNA charged with $2.1 \cdot 10^4$ counts/min of ^{14}C -phenylalanine. Incubation was for 30 min at 37° .

Ribonuclease ($\mu\text{g}/\text{ml}$)	Incorporation from sRNA- phenylalanine- ^{14}C (counts/min)
0	460
0.25	387
0.5	128
1	60
2	10
3	5

Table 3

EFFECT OF PUROMYCIN AND CHLORAMPHENICOL ON
INCORPORATION INTO PROTEIN OF RADIOACTIVITY
FROM ^{14}C -PHENYLALANINE-sRNA BY RIBO-
SOMES FROM RAT HEART MUSCLE

The reaction was carried out as described in methods. Each tube also contained: the concentration of puromycin or chloramphenicol specified; 0.15 mg of *E. coli* sRNA charged with $2.1 \cdot 10^4$ counts/min of ^{14}C -phenylalanine; 200 μg of polyuridylic acid; and ribosomes prepared by Method 1 (48 μg of RNA). Incubation was for 15 min at 37° .

Concentration M	Incorporation of ^{14}C -phenylalanine into protein	
	Puromycin (counts/min)	Chloramphenicol (counts/min)
0	1690	1690
10^{-6}	1765	
10^{-5}	1512	1735
10^{-4}	1330	1700
10^{-3}	783	1710

Comparison with Liver Ribosomes

Ribosomes from rat heart muscle are as effective as those from rat liver in catalyzing amino acyl transfer from sRNA charged with ^{14}C -phenylalanine to protein. Incorporation by ribosomes from either liver or heart muscle: (1) is proportional to the concentration of ribosomal RNA (Figure 3); (2) shows a similar rate and maximum extent of amino acyl transfer (Figures 3 and 4); and (3) shows similar stimulation by polyuridylic acid (Figure 4). After a short lag period (0 to 5 min) incorporation is approximately linear for about 10 min, tending to plateau thereafter (Figure 3).

Figure 3. Effect of ribosomal concentration on incorporation into protein of radioactivity from ^{14}C -phenylalanine-sRNA. The reaction was carried out as described in methods. Each tube also contained: 200 μg of polyuridylic acid; 0.2 mg *E. coli* sRNA charged with $2.1 \cdot 10^4$ counts/min of ^{14}C -phenylalanine; and the concentration of ribosomes specified. The ribosomes were prepared under identical conditions by Method 1; heart ribosomes contained 50.7% RNA; liver ribosomes 56.6%. Incubation was at 37° .

Comparison with Rat Heart Mitochondria

Mitochondria and mitochondrial fragments from rat heart muscle are effective in catalyzing incorporation of amino acid into protein.²⁵⁻²⁹ For that reason the possibility that the protein synthesis observed with ribosomes is actually due to contamination with small amounts of mitochondria, or fragments thereof, needs to be considered. However, incorporation of radioactivity from ^{14}C -phenylalanine into protein by rat heart mitochondria was found to differ in several respects from that observed with ribosomes from the same tissue (Table 4). Incorporation in the mitochondrial system was not stimulated by addition of polyuridylic acid as it is in ribosomal protein synthesis; it was not inhibited by prior treatment, or incubation, with ribo-

Figure 4. Time course of incorporation into protein of radioactivity from ^{14}C -phenylalanine-sRNA. Conditions were the same as described for Figure 3 except that ribosomes, equal to 60 μg of RNA were added to each tube; the concentration of polyuridylic acid, when present, was 200 $\mu\text{g}/\text{ml}$. Incubation was 37° .

Table 4

INCORPORATION OF RADIOACTIVITY FROM ^{14}C -PHENYLALANINE INTO PROTEIN BY MITOCHONDRIA FROM RAT HEART MUSCLE

The mitochondria were isolated, treated with ribonuclease, and incubated as described in methods. Each 1.0 ml reaction mixture contained 1 mg of mitochondrial protein and 0.125 μC of ^{14}C -phenylalanine. Incubation was for 90 min at 37° .

Additions to medium	Incorporation from ^{14}C -phenylalanine into protein (counts/min)
None	945
200 μg polyuridylic acid	812
100 μg ribonuclease	910
10^{-3} M puromycin	95
200 μg chloramphenicol	238
10 μg actinomycin D	288

nuclease in amounts that are 100-fold greater than those that inhibit protein synthesis by ribosomes; and finally, chloramphenicol and actinomycin D inhibit mitochondrial protein synthesis whereas they are without effect on ribosomes. It is important to bear in mind that those properties apply only to mitochondria that have been freed of ribosomal contamination by careful washing and prior treatment with ribonuclease.

DISCUSSION

Ribonucleoprotein particles may be isolated from heart muscle by differential centrifugation after treatment of the tissue homogenate with deoxycholate. The method is similar to that used by Liao and Williams-Ashman¹¹ for the isolation of ribosomes from prostatic tissue, the principal modification being the use of solutions of higher ionic strength (0.1 M KCl). The method yields particles of reasonable purity whose characteristics accord closely with those of ribosomes from other mammalian tissues. Moreover, the ribonucleoprotein particles from heart muscle will incorporate significant amounts of radioactivity into protein when incubated in vitro with sRNA-phenylalanine-¹⁴C in a reaction that is energy dependent, that is inhibited by ribonuclease and puromycin but not by chloramphenicol, and is markedly stimulated in the presence of polyuridylic acid. Once again these are characteristics that conform closely to those described for protein synthesis by ribosomes from other tissues. That incorporation of radioactivity from sRNA-phenylalanine-¹⁴C into protein by ribosomes from rat heart muscle is so markedly stimulated by addition of polyuridylic acid suggests that they may be relatively deficient in messenger RNA.

A possible hazard in the use of the entire 105,000 x g sediment to prepare ribosomes is contamination by fragments of mitochondria or nuclei which are active in protein synthesis. Active particles have been isolated from mitochondria after treatment with digitonin²⁷ or deoxycholate,²⁹ and after sonic disruption.^{27,28} However, these particles possess characteristics that differ from ribosomes. In contrast to ribosomes, incorporation of radioactivity into protein by mitochondrial and submitochondrial particles has not been shown to be stimulated by polyuridylic acid, is at least relatively resistant to inhibition by ribonuclease,²⁷ and is inhibited by chloramphenicol.²⁷ Therefore it would appear that little if any of the amino acid incorporating activity displayed by ribosomal preparations from heart muscle is due to mitochondrial fragments.

The ribosomes from muscle and those prepared in a similar way from liver are quite comparable in their capacity to catalyze protein synthesis from sRNA-phenylalanine-¹⁴C, and this is true whether synthesis is assayed in the presence or absence of polyuridylic acid. There is, of course, no assurance that the isolated ribosomes retain the characteristics they have in vivo. Nonetheless, the quantitative difference in the amount of protein synthesis carried out by liver and muscle in vivo seems not to be related to a difference inherent in the ribosomes from the two tissues but perhaps to the concentration of ribosomes in the tissue or to some other regulatory mechanism.

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COMPUTER APPLICATIONS IN CLINICAL RESEARCH; EXPLORATION OF A MODEL FOR SULFOBROMOPHTHALEIN (BSP) METABOLISM*

By

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The digital computer, with its capacity for compact storage and rapid retrieval of large amounts of data and its ability to perform complex statistical calculations with speed and precision, would seem to have been designed with clinical research in mind. The great variability among patients and the conditions surrounding their illnesses frequently demand the careful evaluation of a large number of cases before valid conclusions may be reached concerning alternative treatment strategies. Clearly, the computer cannot design experiments, or volunteer the types of statistical analysis appropriate for a given set of data presented to it. Nor can data from a poorly planned or carelessly executed investigation be ennobled by passage through the computer's core memory. Nevertheless, with the help of programs written and applied under the direction of competent statisticians, digital computers are being called with increasing frequency to their appropriate role of accomplishing economically and rapidly tasks of computation and data retrieval that might otherwise require weeks or months.

There are several ways in which the computer's capacity for data storage and retrieval may influence the design of clinical research projects. In the first place, there may be a greater tendency for several institutions to cooperate in a given clinical study. The well-defined procedures for recording data that are required for any computer-oriented study should facilitate such cooperation, and the larger volume of data that results seldom presents a problem. In addition, the amount of data obtained from each patient may increase. Not only may a given set of observations be recorded more frequently, but the gathering of a wider variety of information from each patient may enable more than one research goal to be pursued simultaneously.

Pharmacology is among those medical disciplines that soon realized the potential of computer support. In addition to the retrieval and analysis of research data,¹ early applications have included the coding and storage of structural chemical formulas,²⁻⁵ the analysis of x-ray diffraction data,⁶⁻⁹ and the performance of molecular orbital calculations.

The present paper seeks to illustrate how, at an early level of a given investigation, computer-assisted explorations of a proposed biological model may interact with the efforts of laboratory investigators to suggest proximate goals in the laboratory and modification of the model. It is concerned with the initial exploration of a working model of bromosulphalein metabolism. The purpose of such an exploration is to establish the plausibility of the model and, if this is possible, then to direct the laboratory worker's attention to those model parameters to which

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observations made at certain locations in the system may be most sensitive. In all probability we are not using, nor are we at this time trying to determine, the "correct" set of parameters for the model.

PRELIMINARY INVESTIGATION OF A MODEL FOR BSP METABOLISM

Purpose of the investigation. A group of doctors^{10,11} has noticed a difference in the rate of BSP removal from plasma when subjects receiving estrogens are compared with controls. They would like to determine the site or sites at which estrogens exert their influence on BSP metabolism. The status of the theoretical work on this subject is as follows: Segré¹² has expressed BSP removal from plasma by a function employing a small number of parameters. This type of approach provides an economical, quantitative characterization of the net activity of the system. The determination of these parameters for treated and untreated subjects may help to establish the fact that a difference exists and, with further experimentation, should enable the construction of a type of dose-response curve. Since, however, these parameters do not relate directly to the components of an underlying biological model, this approach does not provide the answer being sought by our biological investigators. An extension of the type of work being done by Goresky¹³ to subjects treated by estrogens would provide a more appropriate approach to this problem and is recommended. In addition, the parameters that Goresky has established experimentally for his model may provide some insight into domains of allowable values for parameters or groups of parameters in our model. We should like to determine whether it is worthwhile to supplement these observations at the vascular-hepatic interface with others that would relate to a more comprehensive compartment model, for example one that might support speculation concerning the role of conjugation in BSP transport.

Probably the greatest limitation to our approach resides in the fact that even a crude compartment model requires the specification of a relatively large number of parameters. Thus, experimentation may reveal only the net variation of a group of parameters; i.e., it may not be possible to assign a unique set of values to the model's parameters. In this event it is possible that in the steady-state limit of continuously-administered BSP, our model may reduce to others previously described.¹⁴ On the other hand, even a more detailed model than this, or the others that have been mentioned, would fall short of reflecting the anatomical and physiological complexity of the plasma-hepatic-biliary system. Although the wiser course might be to concede defeat in the face of such considerations, we decided to explore our compartment model with the following objectives in mind: To find a set of parameters that will bring our model into harmony with experimental observations of BSP plasma concentration under various conditions, as well as with observations relating to the percentage of BSP conjugated in bile and in plasma. If this can be done, it is possible, but not established, that the observed maximum rate of BSP transport from plasma to bile may reflect a limiting step in the conjugation process rather than some saturation property of membranes at the hepato-biliary interface. Next to be investigated is the sensitivity of observed parameters, such as BSP concentration and per cent conjugation, to changes in parameters that characterize the model. Since the purpose of this is to provide an insight into experimental procedures that might test the model most effectively, the per cent conjugation of BSP is computed for bile and the hepatocellular compartment, as well as for plasma.

Theory. A four-compartment model is assumed to characterize BSP metabolism. One of these compartments represents BSP excretion to urine at a rate proportional to the plasma concentration of BSP. Another represents BSP excretion to bile at a rate proportional to hepatocellular concentration of BSP. The net rate of BSP transport between the two remaining compartments, plasma and the hepatocellular compartment, is represented as a sum of the transport rate from plasma to the hepatocellular compartment (which is proportional to BSP concentration in plasma), and the transport rate from the hepatocellular compartment to plasma (which is proportional to BSP concentration in the hepatocellular compartment). Conjugated and unconjugated BSP are assumed to be transported independently at all compartment interfaces. In the present model, lymphatic feedback to plasma is ignored. BSP conjugation is assumed to occur in the hepatocellular compartment at a rate that is proportional to the product of the molecular concentrations in that compartment of unconjugated BSP and the conjugant, the molecular species which is to be conjugated with BSP. Although conjugants other than glutathione have been identified, the present model treats the conjugants as a single species. The rate of conjugant replenishment at a given time is assumed to be proportional to the difference between the hepatocellular conjugant concentrations before the administration of BSP and at the given time. The simultaneous, non-linear differential equations that express these assumptions are listed in Table 1; Table 2 and Figure 1 describe the symbols used. All quantities except the conjugant concentration and the various BSP concentrations are assumed to be constant during a given integration of these equations.

Table 1

EQUATIONS CHARACTERIZING MODEL FOR BSP METABOLISM
(See Table 2 and Figure 1 for description of symbols)

$$\frac{dC_P}{dt} = [DRIP + (HP) \cdot C_H - U \cdot C_P - (PH) \cdot C_P]/V_P$$

$$\frac{dC_{JP}}{dt} = [(HPJ) \cdot C_{JH} - (UJ) \cdot C_{JP} - (PHJ) \cdot C_{JP}]/V_P$$

$$\frac{dC_H}{dt} = [(PH) \cdot C_P - \{(BK) + (HP) + (CJCOEF) \cdot C_{JUG}\} \cdot C_H]/V_H$$

$$\frac{dC_{JH}}{dt} = [(PHJ) \cdot C_{JP} + (CJCOEF) \cdot C_{JUG} \cdot C_H - \{(BKJ) + (HPJ)\} \cdot C_{JH}]/V_H$$

$$\frac{dC_{JUG}}{dt} = [(CJSYNT) \cdot (1 - C_{JUG}) - (CJCOEF) \cdot C_{JUG} \cdot C_H]/V_H$$

Computer program. Three successive rates for the intravenous infusion of BSP may be specified, as may the duration of each, in minutes. A single injection is characterized by a one-minute infusion followed by two time periods during which the infusion is specified as zero. The sum of all infusion times must not exceed 200 minutes. This information, together with a specification of the model parameter values, is supplied to the computer for each calculation. There is no limit to the number of calculations that may be performed during a given

Table 2
DEFINITION OF SYMBOLS

DRIP	= intravenous infusion rate for unconjugated BSP (mg/min)
VH	= total hepatocellular volume (ml)
VP	= plasma volume (ml)
PH	= transport coefficient from plasma to hepatic cells for unconjugated BSP (ml/min)
PHJ	= transport coefficient from plasma to hepatic cells for conjugated BSP (ml/min)
HP	= transport coefficient from hepatic cells to plasma for unconjugated BSP (ml/min)
HPJ	= transport coefficient from hepatic cells to plasma for conjugated BSP (ml/min)
U	= transport coefficient from plasma to urine for unconjugated BSP (ml/min)
UJ	= transport coefficient from plasma to urine for conjugated BSP (ml/min)
BK	= transport coefficient from hepatic cells to bile for unconjugated BSP (ml/min)
BKJ	= transport coefficient from hepatic cells to bile for conjugated BSP (ml/min)
CNJANT	= concentration of conjugant in resting hepatic cells (unit/ml "unit" is chosen so that CNJANT = 1 in present model)
CJSYNT	= replenishment coefficient for conjugant (ml/min)
CJCOEF	= conjugation rate coefficient (ml ² /min-unit)
C _P	= plasma concentration of unconjugated BSP (mg/ml)
C _{JP}	= plasma concentration of conjugated BSP (mg/ml)
C _H	= hepatocellular concentration of unconjugated BSP (mg/ml)
C _{JH}	= hepatocellular concentration of conjugated BSP (mg/ml)
C _{JUG}	= hepatocellular concentration of conjugant (unit/ml)

Note: Concentrations of conjugated BSP are expressed as mg/ml of the BSP portion only.

computer run. The initial concentration of BSP in all compartments is assumed to be zero. The units of conjugant concentration are chosen so that the initial concentration is unity.

A Runge-Kutta integration of the simultaneous equations is performed at time intervals of 1 minute. A trial integration at smaller time intervals had little influence on the results and significantly increased the computation time. The printed output provides, at one-minute intervals, total BSP concentrations and per cent conjugation in the plasma and hepatocellular compartments, as well as the amount and per cent conjugation of BSP transported each minute to the

Figure 1. BSP metabolism model. Diagrammatic display of symbols defined in Table 2.

biliary system. Also provided is a list of the specified model parameter values and the BSP infusion schedule. The instantaneous time rate of change of plasma total BSP concentration is calculated at the end of each infusion period, and is compared to the same time rate calculated on the basis of concentrations at the midpoint and end of the period.

Graphical displays of plasma total BSP concentration and of per cent conjugation in plasma, liver, and bile may be provided as an option. These are generated on a cathode-ray tube controlled by the IBM-7094 computer, immediately following the performance of the corresponding calculations. Two types of display may be requested: The first generates a single graph for each of the above quantities as a function of time. The second differs primarily in that each graph displays three curves which compare the results of three calculations; these calculations differ only in the value specified for one of the parameters. Parameter values and the BSP infusion rates are indicated on each graph. Examples of the second type of graphical display are shown in Figure 2, for a case where continuous intravenous infusion has been specified.

RESULTS

A set of parameter values that brought the calculated plasma total BSP concentration curves into correspondence with those observed here for subjects receiving continuous BSP infusion is presented in Table 3. With this choice of parameters, the per cent of BSP conjugated in bile tends toward a plateau exceeding 90 per cent for either continuous intravenous administration or a single injection of BSP. The per cent of BSP conjugated in plasma rises continuously during infusion; but, whereas the low scale of values imposed by requiring HPJ to be small compared to HP is consistent with information on hand at the time of calculation, a relative increase in HPJ is required to achieve correspondence with the values obtained by Schoenfield, Foulk, and Butt.¹⁵ Data presented in Table 4 suggest that this can be done without a loss of the correspondence established with the other experimental observations. This correspondence seems sufficient to suggest that among hypotheses being advanced to explain the maximum rate of transport of BSP from plasma to bile, one should consider the possibility that a limitation in this rate may

Figure 2. Examples of graphical display on cathode ray tube.

Table 3
SET OF PARAMETERS INITIALLY
SPECIFIED FOR MODEL

VP	= 2500 ml
VH	= 1000 ml
PH	= 200 ml/min
PHJ	= 1.8 ml/min
HP	= 20 ml/min
HPJ	= 0.6 ml/min
U	= 0.01 ml/min
UJ	= 0.01 ml/min
BK	= 1.8 ml/min
BKJ	= 18 ml/min
CNJANT	= 1 unit/ml
CJSYNT	= 60 ml/min
CJCOEF	= 25 ml ² /min-unit
<u>Intravenous Infusion Schedule:</u>	
DRIP	= 15 mg/min for 60 min
DRIP	= 9 mg/min for 60 min
DRIP	= 9 mg/min for 60 min

reflect effectively a limitation in conjugation rate because of a greater permeability of the hepato-biliary interface to conjugated BSP.

In order to test this model or, assuming it, to gain insight into experiments that might suggest the site(s) of estrogen interaction with BSP metabolism, one should determine the sensitivity of observed parameters to variations in individual model parameters. These relationships usually depend on the set of values initially selected for the parameters, and therefore a variety of such sets should be explored. Table 4 summarizes the results of such an investigation in the neighborhood of the parameter values listed in Table 3. The left-hand column of the table indicates the parameter varied, and each entry in the table compares curves calculated for three-hour periods of observation, the indicated parameter assuming its initial value and then values 60 per cent to either side of the initial value (30-per cent variations gave results consistent with these). Observable differences are rated on a scale of 1 to 3. The following observations might be made: Several hours after a single injection of BSP, per cent conjugation in plasma is very sensitive to variations in most parameters. The practical exploitation of this observation is limited by the low plasma concentration of BSP at that time. For both infusion and single injection, plasma BSP concentration is especially sensitive to PH, and per cent conjugation in bile responds moderately to liver volume, the conjugation coefficient, and BKJ. Except for changes in BKJ or BK, variations of per cent conjugation in the hepatocellular compartment resemble or amplify those in bile. Observations such as these also may be made with the aid of an analog computer, although direct inspection of the model suffices for many of the qualitative inferences.

Table 4

SENSITIVITY OF OBSERVABLE PARAMETERS TO CHANGES IN MODEL PARAMETERS

	Continuous infusion				Single injection			
	Plasma BSP Conc.	Per cent Conjug. Bile	Per cent Conjug. Liver	Per cent Conjug. Plasma	Plasma BSP Conc.	Per cent Conjug. Bile	Per cent Conjug. Liver	Per cent Conjug. Plasma
VP	-	0	0	-L	--E +M	0	-L	---M,L
VH	-	--E,M	--E,M	-L	-M	-E,M	--E,M	---M,L
PH	---	0	+M,L	+ to ++L	---E,M --L	+M,L	++M,L	+++M,L
HP	++M,L	0	0	-	++M	0	-L	---M,L
PHJ*	0	0	0	0	0	0	0	0
HPJ	+L	0	0	+M,L	+L	0	0	++ to +++M,L
CJCOEF	--M,L	+ to +++	+++	+	-M,L	+ to ++-	+++	+++M,L
BK*	0 to -L	--	0	0	0	--M	0 to +L	0 to +L
BKJ	-L	+ to ++E,M	--M ---L	-L	0 to -L -L	+ to ++E,M	--M ---L	--L
CJSYNT*	0 to -L	0 to -L	+L	0 to +L	0	0	0 to +M,L	0 to +M,L

* Lack of sensitivity to these parameters may be attributable to the small value they assume in the present model.

E,M,L refer to regions of time: early, middle, and late.

+ indicates a positive correlation between observed parameter and model parameter.

- indicates a negative correlation between observed parameter and model parameter.

Magnitude of correlation is rated on a scale of 1 to 3.

If this model is assumed, observation of a subject's biliary and plasma BSP before and during treatment with estrogens might provide the following information: If an increased plasma retention of BSP reflects primarily an alteration in the plasma-hepatic transport mechanism, then associated changes in the per cent of BSP conjugated in bile should be relatively small. If, however, conjugation in mechanisms or a large change in BKJ are responsible, one should be able to detect a decrease in the per cent of BSP conjugated in bile. On the other hand, a noticeable accompanying increase in the per cent conjugation in bile would direct attention to BK or the hepatocellular volume. Observable changes in the per cent conjugation in plasma might help to resolve the ambiguity encountered in either of the last two cases.

A comparison of the removal from plasma of conjugated and unconjugated BSP might test the validity of the proposed model, provided one knew approximately the relative sizes of PH and PHJ. The difficulty of isolating an element of the hepatocellular compartment probably precludes a most valuable comparison between per cent conjugation in this compartment and in bile. If possible, one might explore BSP removal from plasma in the presence of competitors for glutathione conjugation.

The response of the model to a single injection of BSP, using the model parameters listed in Table 3, indicates an abnormally high plasma retention at 45 minutes after injection. Subsequent explorations of the model will be directed to the discovery of a set of parameters that will bring the model into correspondence with experimental observations for both continuous infusion and single injection BSP administration.

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