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ARGONNE CANCER RESEARCH HOSPITAL
950 EAST FIFTY-NINTH STREET • CHICAGO • ILLINOIS 60637

Semiannual Report to
THE ATOMIC ENERGY COMMISSION

MARCH 1965

LEON O. JACOBSON, M.D.
Editor

MARGOT DOYLE, Ph.D.
Associate Editor

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DISEASES OF BONES AND JOINTS ASSOCIATED WITH INTOXICATION BY RADIOACTIVE SUBSTANCES, PRINCIPALLY RADIUM*

By

R. J. Hasterlik and A. J. Finkel†

The history of the use of radium salts intravenously and orally as a medicament during the period 1910-1930 is less well known than the experience of dial painters who acquired radium by occupational exposure. However there still exist in this country many hundreds, perhaps thousands, of persons who carry a significant body burden of radium acquired either from their physicians or in the course of their work. This elevated body content of radium varies from slightly above the normal amount which all of us carry up to a level one hundred thousand times greater.

At the present time, three groups of investigators in the United States, and several others abroad, are engaged in systematically studying as many such persons as can be found. These studies include accurate estimates of the body content of radium by gamma ray spectroscopy and analysis of the expired breath for radon, roentgenographic studies of the entire skeleton, and hematologic examination. These data are used in an attempt to correlate the number and severity of bone lesions, including neoplasia, with the level of retained radium in the body.

Pathogenesis of Bone and Joint Disease

Radium, like calcium and the other divalent cations, is for the most part deposited in the skeleton after it enters the body from the gut. The various details concerning the exact sites of localization within the crystal structure and organic matrix of bone are beyond the scope of this discussion. Briefly, the radium present in the circulating plasma is deposited in those osteons that are being formed at that time. If the radium was administered parenterally by a physician during the course of a few injections, the bone was, as it were, "flash labelled" and the concentration of radium in those newly forming osteons will have been very great. On the other hand, radium acquired over several years' time through oral ingestion, such as was the case with the dial painters, will have been more uniformly distributed throughout a larger number of osteons. Bone turnover, through resorption of old osteons and formation of new ones, subsequently results in a diffuse labelling of most of the osteons at a relatively low concentration by the radium released to the circulation during resorption. Interspersed in this matrix of osteons labelled at low concentration are the unresorbed osteons labelled with a high radium concentration at the time of its acquisition. This process produces the so-called "hot spots," which may contain as much as a 200-fold concentration of radium in comparison with the more uniformly labelled background.[1]

Bone that contains high concentrations of radium 20 to 40 years after its deposition, is for the most part dead bone (Figure 1). The lacunae are devoid of osteocytes. Many of the central

*This report is taken from a paper that appeared in Med. Clin. N. America 49:285, 1965.

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canals of the haversian system are plugged with a dense hydroxy-apatite-like material,[2] a situation resulting in a marked decrease in number of patent central arterioles. The orderly process of bone resorption and new bone formation is disturbed. Osteoclastic activity is not followed by osteoblastic activity in normal sequence and large resorption cavities filled with a gelatinous material or an osteoid-like matrix are formed. These areas range in size from that of an osteon up to several centimeters in diameter.

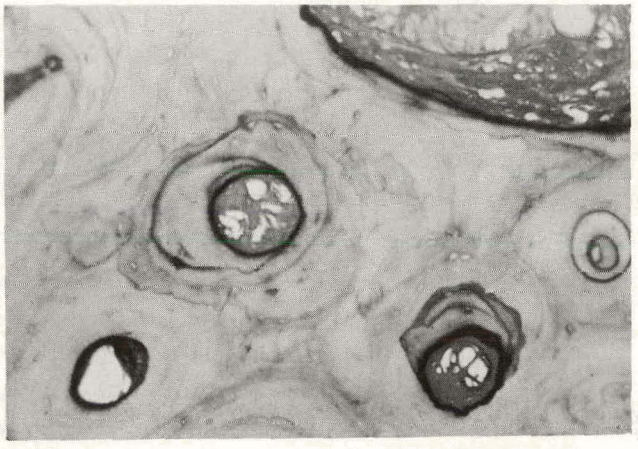

Figure 1. Photomicrograph of bone from patient bearing a body burden of 2.5 μc of Ra^{226}. Note dense calcium-containing plugs occluding the central canals of the haversian systems, absence of osteocytes in lacunae, and the very small and very large resorption cavity.

All cells embedded in the bone in these radium-containing patients and many of the cells of the periosteum and endosteum are continually exposed to irradiation by the alpha and beta particles and x-rays produced by the radioactive disintegration of radium through its decay chain to its final stable daughter, Pb^{206}. This continuous irradiation may lead to tumors of the mesenchymal cells of bone (sarcomata) and also to tumors of the epithelium directly contiguous with bone (carcinomata). The latter situation is found in the paranasal sinuses, the mastoid air cells, and the alveolar ridge of the maxilla. The pathogenesis of these radiation-induced tumors is beyond the scope of our interest in this discussion, and so will not be elaborated on here. However, the relatively high levels of bone irradiation occurring at early times after radium acquisition followed by the many years of lower radiation dose produce bone and joint changes that resemble those of other disease processes.

Roentgenographic Findings

The major bone and joint changes occurring in these radium-bearing patients have been described in detail by Martland,[3] Aub, et al.,[4] Looney, et al.,[5] Hasterlik,[6] and others. Taken individually the roentgenographic findings are not unique for radium poisoning, and they accurately mimic those of many other diseases, especially those related to vascular lesions of bone. Nevertheless, a characteristic pattern of bone and joint lesions is seen in these radium patients and permits a presumptive diagnosis of radium poisoning before the presence of radium has been demonstrated by physical determinations. Moreover, the roentgenographic pattern of bone and joint lesions and their number and severity allow those familiar with this condition to make es-

timates of the body content of radium that are very close to the value actually determined.

The patients studied during the past 15 years were about 20 to 40 years of age when they acquired their radium and approximately 30 to 40 years have now elapsed since exposure. For the most part the dial painters were young; many were high school girls and a large number were 17 to 22 years old. Patients that received radium as a medication or as a tonic ranged from childhood to middle age at the time of treatment. The pertinent data concerning the patients whose radiographs are discussed here are given in Table 1.

The minimal roentgenographic lesions that are typical of radium deposition consist of areas of bone resorption located in the cortices of the shafts of the radius, ulna, tibia, and fibula. These areas may vary from 1 to 2 mm up to 10 mm or more in diameter. In addition, small "punched out" areas of bone resorption of comparable size may also be present in the calvarium (Figure 2). The small areas of bone resorption represent an extension of the process demonstrated microscopically in Figure 1. These lesions may increase in size and number with the passage of

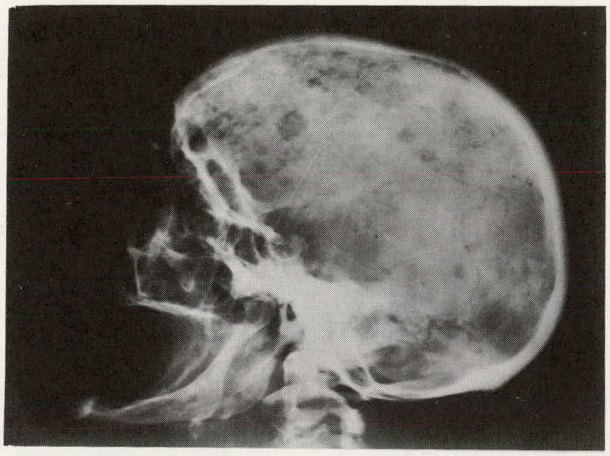

Figure 2. Patient 03-484 (Table 1). Note small and large areas of bone resorption in the calvarium which closely resemble those of myeloma or metastatic malignancy.

time and may also be seen in more severe cases in the humerus, femur, and pelvis. Concomitantly one may see areas of bone sclerosis in the metaphyseal region of the long bones. In addition to the areas of resorption, parametaphyseal sclerosis is commonly seen in those patients who were very young adults with still active epiphyses at the time of radium ingestion (Figure 3). Frequently, especially at higher dose levels, bone resorption is more extensive, and in these cases larger and more numerous lesions are present. The radiograph illustrated in Figure 4 is typical of this more advanced stage. Certainly this appearance could be simulated by hyperparathyroidism and metastatic malignancy.

Also frequently seen in these patients is the marked coarsening of the trabecular pattern of the bone, somewhat resembling that seen in Paget's disease or after long term administration of corticosteroids. This change is the result of resorption of the finer trabeculae so that the remaining heavier trabeculae present a picture of residual coarsening.

Of especial interest in differential diagnosis are those lesions that appear as areas of dense bone sclerosis, and which simulate bone infarcts secondary to other etiological factors. It would

Table 1

SUMMARY OF CASE HISTORIES

Figure No.	Code No.	Date of Birth	Source of radium	Body burden of Ra^{226}		Comments
				Date	Amount	
7	03-404	1897	Dial painter, 1923-1927. Tipped brush between lips.	1957 1962	0.98 μc 0.86 μc	Musculoskeletal complaints confined to low back discomfort after shoveling snow, December, 1961.
3	03-455	1906	Dial painter, 13 months, 1922-1923. Tipped brush between lips.	1958	0.81 μc	Fibrosarcoma, head of left radius; treated successfully by disarticulation of left upper extremity in 1934.
5,8	03-473	1904	Dial painter, 2 years, 1922-1924. Tipped brush between lips.	1958 1962	1.26 μc 1.17 μc	Severe, progressively disabling arthritis of hips, starting after pregnancy in 1938.
2	03-484	1888	Dial painter, 3 years, 1919-1922. "Never put brush in mouth."	1958 1962	1.55 μc 1.44 μc	Medical history negative until duodenal ulcer discovered in 1959. Mild left hip pain started in October, 1962.
6	03-209	1895	Received radium by injection from personal physician, 1925-1926, for treatment of psoriasis.	1951	1.0 μc	Painful right elbow, 1950; x-rays revealed aseptic necrosis of head of radius. Painful shoulder, September, 1958. Biopsy of right acromion process demonstrated an anaplastic sarcoma. Died of widespread metastases, 1960.
4	03-226	1875	Physician who received intravenous injections weekly for nine months in 1934 for vague arthritic pains in both shoulders.	1951	10.7 μc	Loss of all teeth and anterior half of mandible in 1936. Stiffness of right hip developed in 1949. Anemia treated by transfusions in 1951. Died in 1953 of aplastic anemia.

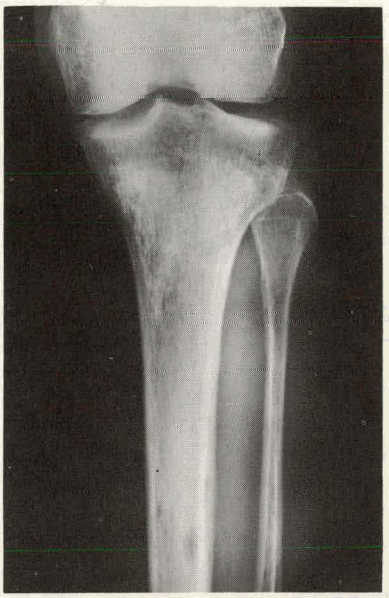

Figure 3. Patient 03-455 (Table 1). Note parametaphyseal bone sclerosis in patient who ingested radium in young adult life. Also note multiple areas of bone resorption in cortex of the fibula and tibia.

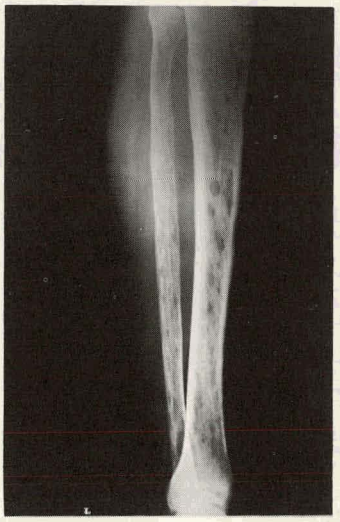

Figure 4. Patient 03-226 (Table 1). Multiple large and small areas of bone resorption with marked coarsening of the trabecular pattern. Marked similarity of appearance with bone lesions of hyperparathyroidism and metastatic malignancy.

be hard to distinguish the sclerotic lesions in the radium-bearing patient shown in Figure 5 from those of caisson disease. These areas of sclerosis are common in our patients and are most frequently seen in the head and neck of the humerus and femur, in the epiphyseal regions of the extremities, and in the pelvis. The pathogenesis of these lesions is probably similar to those in which there is clear-cut evidence for vascular occlusion. However, instead of acute vascular occlusion of small vessels in the bone from such agents as nitrogen bubbles, here the occlusion must represent radiation damage to vessel walls occurring over a prolonged period of time as

well as injury resulting from plugging of the central canals of the haversian systems.

Further important items in differential diagnosis are those lesions of bone that occur in adult life in radium-bearing patients, and that are otherwise more commonly seen in the child or adolescent. These lesions include aseptic necrosis of the head of the humerus, femur, radius, and tarsal scaphoid. Figures 6 and 7 demonstrate typical examples in radium-bearing patients. The pathogenesis is uncertain but again it would seem that some change in the normal vascularity of the region must be involved.

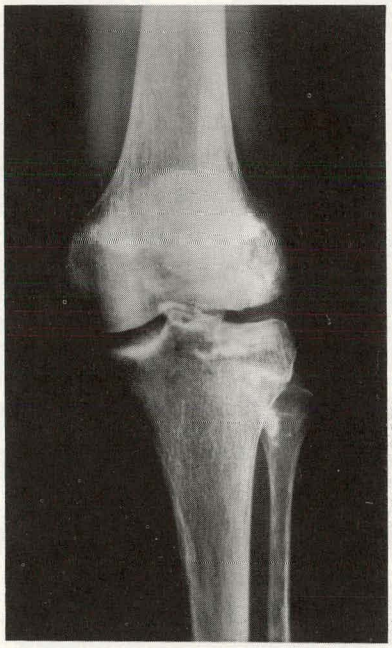

Figure 5. Patient 03-473 (Table 1). Sclerotic lesions of femur, tibia, and fibula simulating those of caisson disease.

Figure 8 illustrates an advanced stage of radium-related bone and joint changes. Note especially the areas of bone resorption in the shaft of one femur, the coarsening of the trabecular structure throughout, the areas of bone sclerosis in both femurs, and the aseptic necrosis and destruction of the heads of both femurs. The number and severity of these lesions are related to the magnitude of the body content of radium remaining many years after the initial deposit of the radioactive material. Such changes are seen not only in the aged, but also in some radium patients as early as the 40-year-age group. Without doubt, when radium is present the usual changes of hypertrophic osteoarthropathy are exaggerated. On the other hand, the concomitant appearance of many of the "hallmarks" of radium deposition gives confidence in the view that the patient does in fact contain radium and that the observed changes are causally related to its presence.

Pathological fractures are also seen in advanced cases in addition to the aforementioned coarsening of trabeculation, localized areas of bone resorption, spotty sclerosis, and aseptic necrosis. These fractures most frequently affect the femur, humerus, and patella, and have occurred in the absence of injury or with only trivial stress. Interestingly enough, with proper alignment and fixation these fractures heal well.

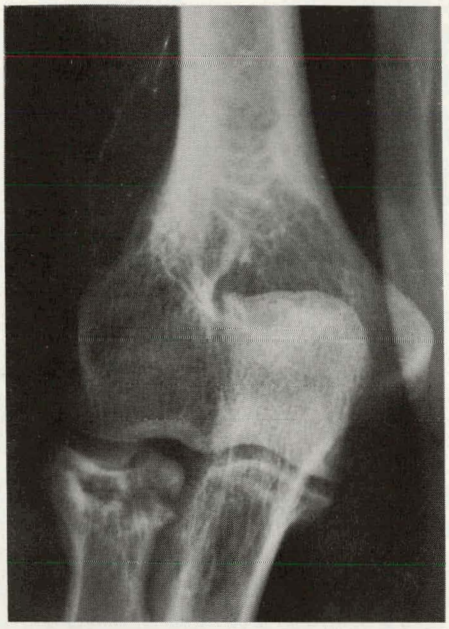

Figure 6. Patient 03-209 (Table 1). Aseptic necrosis of head of the radius, occurring spontaneously and in the absence of trauma. From Looney, Hasterlik, Brues, and Skirmont, The American Journal of Roentgenology and Radium Therapy 73(6):1006, June, 1955. Courtesy of Charles C. Thomas, Publisher, Springfield, Illinois.

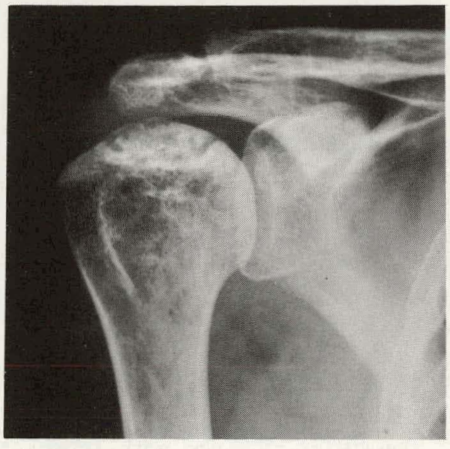

Figure 7. Patient 03-404 (Table 1). Aseptic necrosis of the head of the humerus occurring spontaneously in a radium-bearing patient.

Neoplastic Changes

Especially well known among the neoplasms seen in these patients are the sarcomata arising in the skeleton and well described by Martland and Humphries,[7] Aub, et al.,[4] and Looney, et al.[5] These sarcomata may or may not form new bone. Most frequently they resemble fibrosarcomata or spindle cell sarcomata. They may arise in the mesenchymal cells of any of the bones, and not necessarily in areas of apparent bone necrosis. Not infrequently they are multicentric in origin. As is typical of other examples of osteosarcomata, these tumors metastasize widely.

Less well known are the carcinomata that arise in the epithelium lining the mastoid air cells, the paranasal sinuses, and the mouth, especially in the alveolar ridge of the maxilla. These carcinomata are being seen with increasing frequency, although they were reported by Aub, et al. in 1952[4] and more recently by our group.[8] An unusual tumor of mesothelial origin (rhabdomyosarcoma) has been observed in the maxillary antrum of one of our patients.

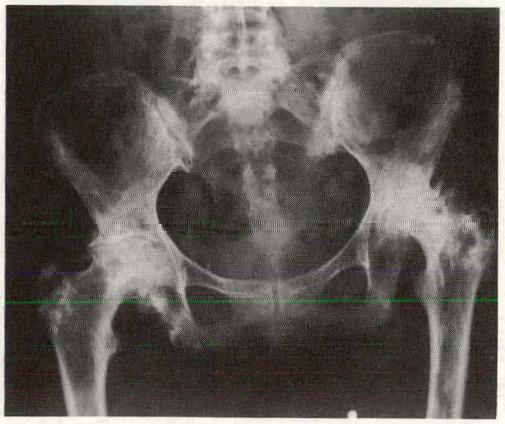

Figure 8. Patient 03-473 (Table 1). Multiple advanced orthopedic lesions of radium poisoning. Note multiple areas of bone resorption in the femur, coarsening of the trabecular pattern throughout, bone sclerosis, and aseptic necrosis of the heads of the femurs.

Must we postulate a special radiosensitivity of these particular tissues to account for the induction of these rare epithelial tumors? The authors believe that epithelial or mesothelial tumors may be seen at any site where a very thin epithelium or connective tissue lies in immediate apposition to the radium-containing bone. It is in the sinuses, mastoids, and tooth sockets that these particular anatomical relationships exist and make possible the induction of these very rare tumors.

Many of the patients who were given radium salts orally or parenterally by physicians in the period from 1910 to 1930 were unaware that they were given radium, or have since forgotten. There is evidence that radium was administered to many thousands of people in this country during that period.[9] Hundreds of them may still be alive since it is known that radium was given orally or parenterally in many non-malignant conditions such as psoriasis, psychoneurosis, vague arthralgias, etc.[10] The clinician faced with atypical bone necrosis, pathological fractures, unusual tumors about the head and neck, and osteosarcomata appearing in late adult life should be alert to the possibility that these may be related to the presence of radium in the skeleton. Appropriate physical studies, such as gamma ray spectroscopy with large scintillation crystals, can then easily establish the presence or absence of this radionuclide.

Intoxication with Other Radioelements

Until this point we have limited our discussion to those bone and joint changes related to the presence in the skeleton of significant quantities of radium (Ra^{226}). Certain other radioactive substances theoretically possess the potentiality to induce these same types of tumors and to cause similar bone and joint changes. These bone-seeking radionuclides include radiothorium,

mesothorium, plutonium, and radioactive strontium (Th^{228}, Ra^{228}, Pu^{239}, Sr^{90}). Radiothorium and mesothorium are known to have been used with radium in some of the luminous paints in the dial industry, and undoubtedly were involved in the production of tumors and bone lesions in some of these patients. On the other hand, because of the high degree of protection afforded the worker in the various Atomic Energy Commission enterprises, no cases of bone pathology or bone tumor have been seen that can be causally related to the presence of Sr^{90} or Pu^{239} in the skeleton. These latter substances do not represent a practical problem to the physician in today's circumstances. Certain speculative extrapolations have concluded that a certain number of bone tumors may be related to the fallout of Sr^{90} from nuclear weapons tests. Other theoretical considerations as well as some experimental studies lend weight to the argument that the amount of Sr^{90} present in our skeletons from fallout probably is not carcinogenic. At this stage it is not possible to give a definitive answer to this question.

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A RADIOISOTOPIC DEVICE FOR MEASURING BONE MINERAL*

By

L. H. Lanzl, and N. M. Strandjord†

The physician can tell a great deal about the health of a patient from the condition of his blood. Tests of the blood are performed by taking a small sample, usually a drop or two, which serves to represent the condition of the entire blood volume of the patient. The situation with respect to skeletal bone is entirely different. The physician and the patient are reluctant to have a sample of bone removed for study and testing.

An instrument using radioactive iodine-125 has been devised for non-destructive testing to determine the condition of the bone mineral in the skeleton. This is accomplished by studying the transmission through a single finger bone of the radiation emanating from iodine-125. For small-animal work, a rear leg is used. The smaller the bone mineral content, the higher will be the transmission of the radiation through the bone. A lower value of bone mineral content may be due to a thinner bone, a bone of lower density, or a combination of both. A change in the effective atomic number of the bone will also result in a change in the radiation transmitted through it.

The use of transmission measurements for analyses of one sort or another has been employed very often in physics. In the present context, the monograph of Omnell[1] contains a good summary of the work done before 1957 on bone mineral changes in vivo. Omnell reports that, in general, the methods employed a standard x-ray tube as the radiation source and photographic film as the detector.

Our work has been stimulated by that of Cameron and Sorenson[2] at the University of Wisconsin, and we have followed several of their ideas. Cameron and Sorenson state that some of the ways in which their method differs from earlier methods are:

(i) the transmission of the photon beam is measured directly by counting techniques, employing a scintillation detector system;
(ii) the photon beam used is essentially monochromatic;
(iii) the photon beam and detector are well collimated; and
(iv) the effects of the tissue around the bone are taken into account.

In the present work, the measuring unit was designed primarily to accommodate a phalanx as a skeletal sample. In particular, the second phalanx of the third digit on the left hand was chosen, since the overlying soft tissue is at a minimum, and immobilization during measurement is easily attained. Thus, correction factors and errors due to the presence of soft tissue are at a minimum. It is true that the lumbar spine, pelvis, and os calcis show a greater degree of demineralization than the small bones of the hand, but technical considerations make estimates

*Presented at the SYMPOSIUM ON LOW ENERGY X AND GAMMA RAY SOURCES AND APPLICATIONS, which was sponsored by the Illinois Institute of Technology Research Institute and the United States Atomic Energy Commission on 20-21 October 1964.

†Department of Radiology, University of Chicago, Chicago, Illinois.

of mineral content of the spine or pelvis difficult. Cameron and Sorenson have used the radius of the left arm. Here, the bone is more massive, but the overlying tissue is somewhat greater than in a finger. Virtama, et al.,[3,4] Williams and Mason,[5] and other investigators have used a finger. In consideration of the above, we chose the finger as a means to measure mineral content.

A study of the absorptive characteristics of muscle and bone tissues in humans or animals by x- or gamma radiations reveals that the greatest discrimination occurs between 20- and 30-kilovolt energy photons.[6] This is shown in Figure 1. Assuming an effective atomic number of

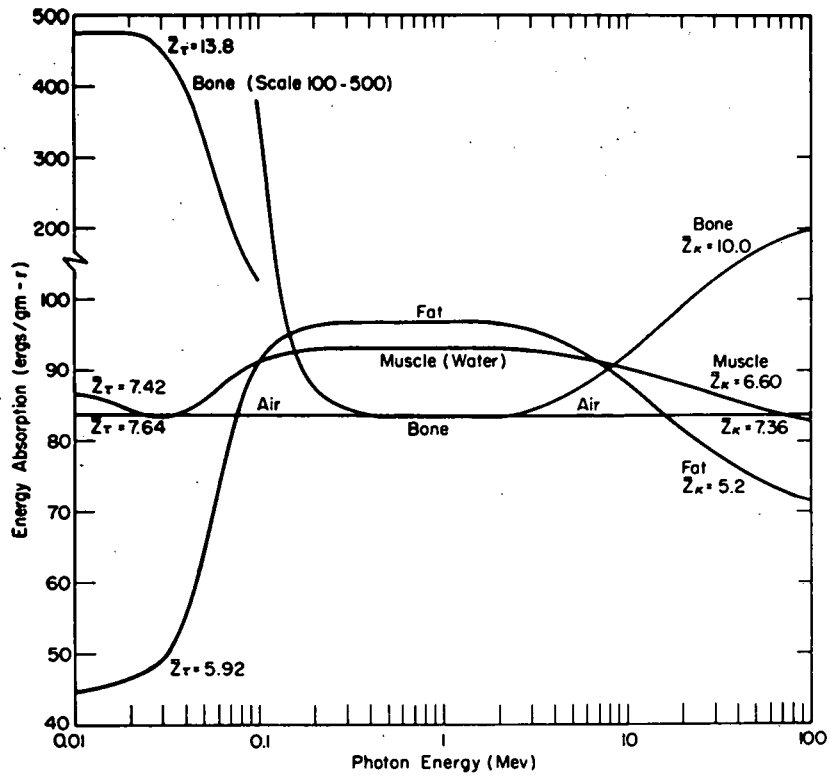

Figure 1. Interaction of radiation with matter (reproduced by courtesy of Radiation Dosimetry, 1956 edition, p. 90, published by Academic Press Inc., New York).

13.8 for bone, and 7.42 for muscle, the energy absorption is about 460 and 83.9 ergs/gm-r for bone and muscle, respectively. From these figures, we see that the ratio of energy absorbed per gram of tissue is $\frac{460}{83.9} = 5.5$ for bone to muscle. The ratio of bone to fat is still greater. When scattering as well as absorptive characteristics are taken into account, still greater differences for bone to muscle are observed and expected in a "good" geometry situation, which is the case for these transmission measurements. While a low-energy x-ray machine is capable of producing photons in the range of 20 to 30 kilovolt energy, it produces in addition many photons outside this range.

Furthermore, the intensity of an x-ray machine is not inherently stable. On the other hand, the time rate of output of a radioactive isotope of sufficiently high strength is very stable, although it does decay.

A particularly useful isotope for this purpose is iodine-125, which decays by means of electron capture to a 0.354 MeV excited state of tellurium-125. From this state, tellurium decays by means of a gamma ray which is 80 per cent internally converted in the K shell, giving, in turn, two x-ray photons, K_α = 27 keV and K_β = 31 keV.[7] There are several other radiations such as the characteristic x-rays from the L, M and N shells resulting from electron capture, conversion electrons and Auger electrons, but their energy or range is such that they do not play a role in the present work.

Of the three photons, the K_α = 27 keV is the most abundant, accounting for 75 per cent of these photons per disintegration. The K_β accounts for 20 per cent of the total, only 5 per cent remaining as unconverted 35.4 keV gamma-ray transitions.

METHOD

Consider the cross section of a finger which has been compressed between two parallel planes, V and U, as shown in Figure 2. A collimated beam of x- and gamma rays as described above is directed along path S. The transmitted intensity, I, of the beam will equal the unatten-

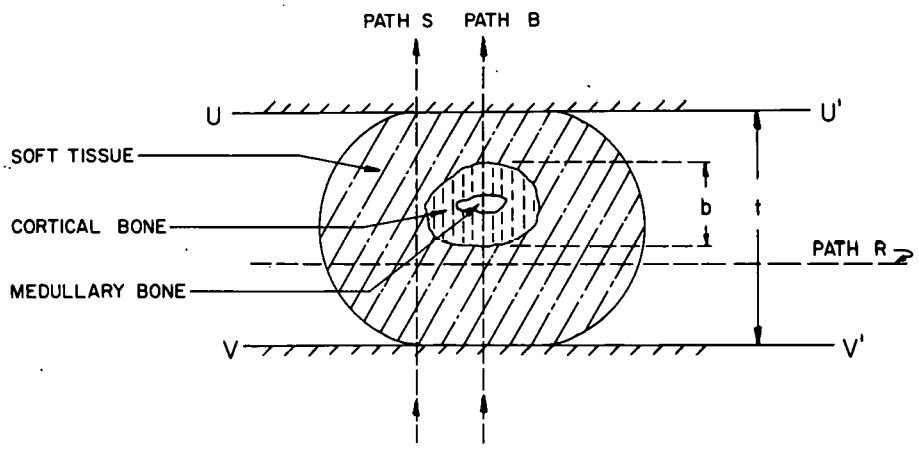

Figure 2. Cross section of a finger showing relationships of bone and soft tissue, and paths of radiation beams.

uated intensity, I_o, times the attenuation, $e^{-\mu_T t}$, of the beam due to the soft tissues, where μ_T is the linear absorption coefficient in cm^{-1}, and t is the thickness of the soft tissue in cm. This thickness is also the distance between the parallel planes V and U. I_o is measured by simply removing the finger from its position between the two planes. From measured values of

$$I, I_o, t$$

and the use of expression

$$I = I_o e^{-\mu_T t}, \qquad (1)$$

the linear absorption coefficient, μ_T, of the soft tissue is obtained. In actual practice, it is a little more reliable to rotate the finger through 90° between the planes V and U, and to make the measurements along path R.

When the beam is directed along path B, the expression contains another factor due to the absorption of the radiation in the bone. This expression is

$$I = I_o e^{-\mu_T (t-b)} e^{-\mu_B b}, \qquad (2)$$

where μ_B is the linear absorption coefficient of bone in cm^{-1}, and b is the thickness of the bone in cm.

The thickness b is obtained from measurements of a radiograph of the bone in question. The radiograph also serves to identify the portion of the bone where the measurement is made. A slotted device, called a finger locator, is calibrated in millimeters and is used to measure the distance from the end of the finger to the point in question. This device can be seen in Figure 3, which also shows a typical radiograph of a finger.

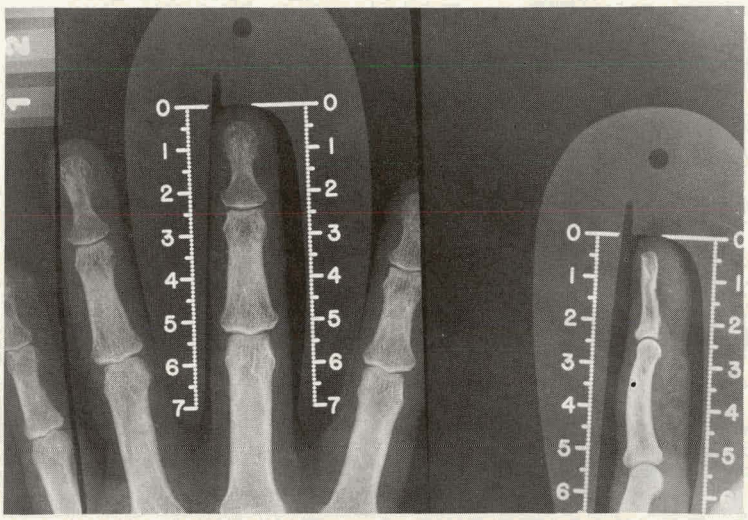

Figure 3. Typical radiographs of fingers with calibrated locator.

The over-all absorption coefficient, μ_B, of the bone is different from the absorption coefficient of the medullary and cortical bones. The expression which permits the evaluation of the absorption coefficients of medullary and cortical bone separately is

$$I = I_o e^{-\mu_{MB} m} e^{-\mu_{CB} (b-m)} e^{-\mu_T (t-b)}, \qquad (3)$$

where μ_{MB} is the absorption coefficient in medullary bone in cm^{-1},

μ_{CB} is the absorption coefficient of cortical bone in cm^{-1}, and

m is the thickness of the medullary bone in cm.

The other terms are defined as above. The thickness of medullary bone is found by means of the radiograph. If a value of μ_{MB} is determined separately, the absorption coefficient of cortical bone can be determined.

Bone consists of supporting tissue plus hydroxyapatite (effective atomic number 16.65). If we take a layer of hydroxyapatite, or material having the same effective atomic number and of a thickness that gives the same attenuation in the iodine-125 beam as is given by an unknown bone in vivo, the density of the in vivo bone can be determined. This assumes that the mass absorption coefficient, $\frac{\mu_H}{\rho_H}$, equals that of the bone, $\frac{\mu_B}{\rho_B}$, and that there is negligible absorption of the radiation in the supporting tissue.

INSTRUMENTATION

The iodine-125 is taken up from a solution by a small amount of ion-exchange resin which is then used as the source. This iodine-containing resin is placed in a small, cylindrically shaped plastic capsule, and is sealed by means of an "O" ring and a thin plastic cover plate. The cylinder is placed in a brass source shield with a sliding shutter which is controlled by an electromagnet, as shown in Figure 4. The beam, as it leaves the source shield, is collimated by an insert of brass. Beam diameters from 0.3 to 2.0 mm have been used.

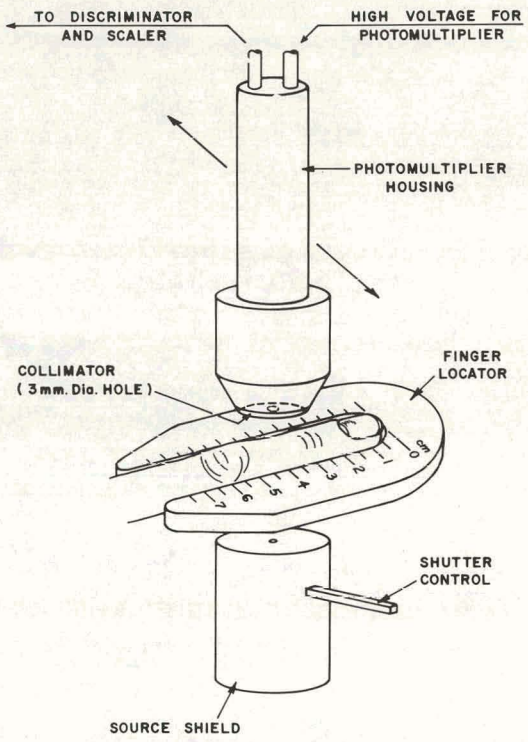

Figure 4. Diagram illustrating the position of finger locator, radiation source, collimator and photomultiplier.

The object to be measured, i.e., finger, is held immobile by means of two plastic plates. The plates are milled out in the region of measurement, but are quite thick elsewhere. These plates are shown in Figure 5.

The beam is detected by a thallium-activated, 2-mm-thick sodium iodide crystal. To prevent light and moisture from entering the crystal, it is covered on the entrance side by a 0.13 mm beryllium foil. This foil absorbs only about 1 per cent of the radiation because of its small

thickness and low atomic number. Since the photon energies from iodine-125 are quite low, the crystal is a very efficient detector from which little energy escapes. The crystal is surrounded by another shield to reduce the background radiation which reaches the crystal. This shield contains a collimator hole 3 mm in diameter (see Figure 4).

Figure 5. Subject with finger immobilized between plastic plates for counting. Data record appears on paper tape at lower right.

The light pulses which result when the x- and gamma rays interact with the crystal are picked up by a photomultiplier. The photomultiplier is followed by a preamplifier, amplifier, and by a single-channel analyzer which actually operates as a discriminator. All pulses below a preset pulse-height level are rejected: All pulses above that level are sent to a scaling circuit to be counted. A differential pulse height spectrum is shown in Figure 6. A Baird-Atomic Cambridge Series Model CS107 scaler and Cambridge Model CS905 timer are used. The unit can be set to count for a preset time, or a preset number of counts. The apparatus is shown in Figure 5. To facilitate data recording, the total counts, if the counting time is preselected, or time, if the total counts are preselected, is automatically recorded on paper tape from a Hewlett Packard Digital Recorder. The power supply for the photomultiplier is a John Fluke Model 405.

The entire unit is normally programmed to make transmission measurements across the entire width of the finger automatically. The finger is held immobile, but the source and detector are mounted on a frame which is driven laterally across the finger by means of a drive motor coupled through an electric clutch and brake to a lead screw. Measurements are made at one millimeter intervals. During each measurement period, the source and detector are held fixed. The electric brake is energized to insure that the lead screw is not rotated, thus preventing the source and detector from moving. Following a counting interval, the brake is de-energized and the clutch is energized, allowing the motor to turn the lead screw one revolution. One

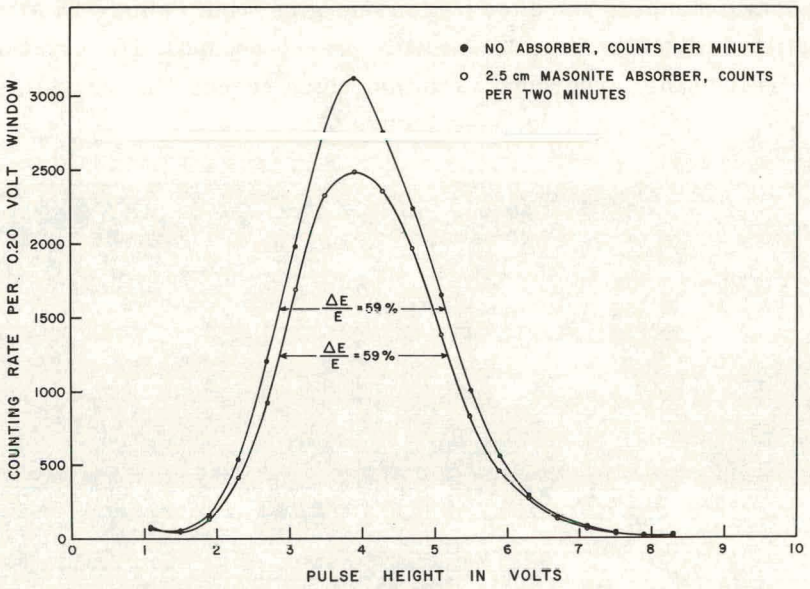

Figure 6. Differential pulse height spectrum of iodine-125. Pulse height vs. counting rate.

revolution corresponds to a one-millimeter movement of the source-detector carriage. During this off period, the digital recorder is activated and prints out the time or the counts for the previous interval. Upon completion of the one-millimeter movement, the measurement mode again takes over. This process is continued until the entire finger has been measured.

DATA HANDLING

Equation (2) relates the initial intensity of the beam to the intensity as altered by the presence of the finger. Solving this equation for the absorption coefficient, μ_B, gives

$$\mu_B = \frac{\ln I/I_o + \mu_T(t-b)}{b} \tag{4}$$

where the terms of the equation are as defined above. The right-hand side of the equation contains five independently observed quantities. It is useful to consider the measurement uncertainties of each of these quantities, and to establish their relationship to the uncertainty of the absorption coefficient of bone. The fundamental formula of the propagation of errors can be used:[8]

$$\sigma_f = \left[\left(\frac{\delta f}{\delta x_1}\right)^2 \sigma_1^2 + \left(\frac{\delta f}{\delta x_2}\right)^2 \sigma_2^2 + \ldots + \left(\frac{\delta f}{\delta x_n}\right)^2 \sigma_n^2 \right]^{1/2}, \tag{5}$$

where σ_f is the standard deviation of a function f of the independently observed quantities x_1, x_2, ... , x_n with their respective standard deviations σ_1, σ_2, ... , σ_n.

Considering the dependent variable to be μ_B from Equation (4), one obtains

$$\sigma_{\mu_B} = \left[\left(\frac{1}{bI}\right)^2 \sigma_I^2 + \left(-\frac{1}{bI_o}\right)^2 \sigma_{I_o}^2 + \left(\frac{t-b}{b}\right)^2 \sigma_{\mu_T}^2 + \left(\frac{\mu_T}{b}\right)^2 \sigma_{t-b}^2 \right.$$

$$\left. + \left(-\frac{\ln I/I_o + \mu_T(t-b)}{b^2}\right)^2 \sigma_b^2 \right] 1/2 \qquad (6)$$

where σ_I, σ_{I_o}, σ_{μ_T}, σ_{t-b}, and σ_b are the individual standard deviations of the quantities I, I_o, μ_T, (t-b), and b.

If a series of measurements of the above quantities is made on an individual and the standard deviations are calculated, a statement can be made about the standard deviation of computed values of μ_B from the mean value, using Equation (6). This standard deviation enables us to distinguish between the uncertainty of an individual determination and a spread of values in a population of persons. We find that it is possible to obtain values of σ_{μ_B} to about ± 1 per cent when each of the individual quantities is carefully measured. In the quantities I and I_o, a sufficiently high number of counts must be taken and corrected for background counts.

TYPES OF EXPERIMENTS

At the present time, we are using the above instrument for several experiments. None of these is complete, but perhaps a few comments on them are in order.

The first involves osteoporosis in postmenopausal females. Osteoporosis is characterized by a decrease in bone mass with normal mineral content. It occurs as a result of varied pathogenic conditions and is associated with a number of different disease processes. Cook[9] collected 800 cases of osteoporosis from the literature and determined the sex incidence to be 6 times higher in females. This leads, for example, to the high incidence of broken hips among elderly women.

With Drs. Edward Davis and Edward Person of the University of Chicago, we have undertaken the study of possible beneficial effects of estrogen therapy in the postmenopausal female. Previous investigators have believed that the anabolic effect of estrogens and androgens will halt osteoporosis when used after its development. However, no objective proof of the beneficial effect of hormones on osteoporosis is available. It is fortuitous that we have a unique group of patients from Chicago Lying-in Hospital who have had bilateral oophorectomy and who are endogenously estrogen deficient. These patients have all received exogenous hormone therapy in the postoperative period. All have been placed on one-half mg of diethylstilbesterol three times a week. We propose to measure the bone content of these patients, and to compare them with postmenopausal females who have not had their ovaries removed and are not on hormone therapy. Measurements on the bones of females in the premenopausal era serve as controls.

Figure 7 is a plot of data taken on about 90 patients in three groups: pre-menopausal, post-menopausal without hormone therapy, and post-menopausal with hormone therapy. The plot is of linear absorption coefficient vs. age of the patient. With careful measurement, the linear absorption coefficient of over-all bone can be determined to an accuracy of 1 or 2 per cent. In the pre-menopausal group, the standard deviation is calculated to be ± 8 per cent. For the post-menopausal group without hormone, it is ± 19 per cent. For the post-menopausal group with hor-

mones, it is ± 14 per cent. The measured data thus are sufficiently accurate to show the variation of a group of individuals. The mean values which appear on the left side of the figure indicate a difference between the pre-menopausal and post-menopausal women which is not at variance with other types of information. The group receiving hormone therapy appears to have a linear absorption coefficient halfway between the other two, indicating a beneficial result as far bone mineral content is concerned.

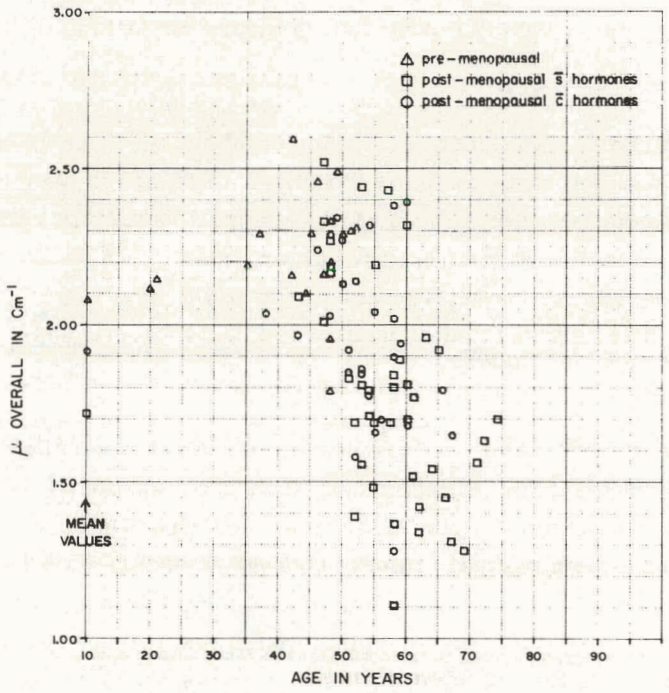

Figure 7. Over-all bone density coefficients of pre- and post-menopausal patients. December 1963 - July 1964.

In another study, we are following closely several individuals with acute diseases. One of these is a female patient who has acute osteomalacia. She is 26 years old and has lost about two inches in height in about two years. We find, on periodic measurement, a decrease of transmission of 8 per cent following a course of hormone therapy. This decrease is significant when compared with transmission measurements through an aluminum standard, and indicates an increase in calcium content. We hope to continue measuring her as the progress of the disease and treatment continue. The result thus far is a hopeful sign that the therapy regimen is a correct one.

In another study, in collaboration with Drs. Paul V. Harper and Edward Paloyan of the University of Chicago, we are measuring the linear absorption coefficient in the leg of a rabbit with an enriched cholesterol diet. Figure 8 is a radiograph showing the leg that is to be measured. In the rabbit there is a single leg bone, the tibia, at the point chosen for measurement, so that there is no risk of measuring a different bone. Figure 9 shows a rabbit in the measuring facility with its leg extended and immobilized for counting. In a first run of three test and three control rabbits, one of the test rabbits exhibited a rising cortical bone coefficient, although the

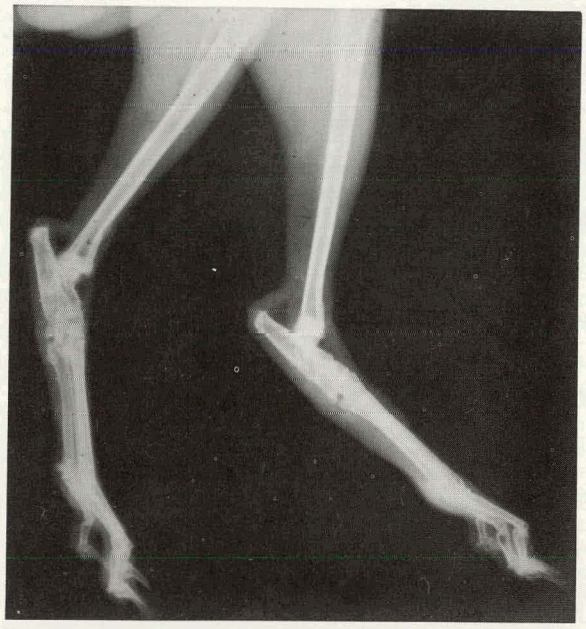

Figure 8. Radiograph of tibia and foot of rabbit. Lateral view.

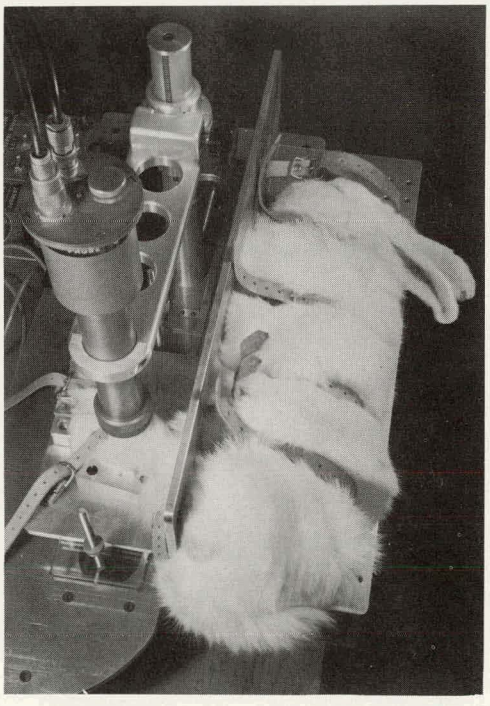

Figure 9. Rabbit with leg extended and immobilized for counting.

bone had not increased in size more rapidly than those of the control rabbits. This rise was not expected since rabbit leg bones become quite brittle with a high cholesterol diet.

ACKNOWLEDGMENTS

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PHYSIOLOGICAL STUDIES OF PRIMITIVE HEMOPOIETIC CELLS*

By

C. W. Gurney, D. Hofstra, and A. Mangalik

The current interest in undifferentiated hemopoietic cells is directly related to the numerous techniques developed during the last five years. These studies hold great promise for clinical medicine since many of the questions posed by the hypoplastic anemias, polycythemia vera, aplastic anemias, and the leukemias may be answered only when the problems of differentiation and the regulation of the numbers of primitive hemopoietic cells are elucidated.

Our interest in these problems originated with our earlier work on red cell production. We have previously demonstrated that red cell production in the mouse can virtually be eliminated by transfusion, and can then be initiated quantitatively by the injection of a single small dose of erythropoietin.[1,2] This approach may then be considered analogous to the "stop-flow" techniques developed by Malvin and Wilde[3] for the study of the physiology of the renal tubule. By further employing the anti-erythropoietin of Schooley and Garcia[4,5] it should then be possible to have a "stop-flow-stop" system, or a model in which a stimulus for differentiation can be applied in vivo for any small predetermined time interval.

Whether or not erythropoietin acts upon normoblasts, there can be little doubt that it exerts a profound effect on primitive cells of the bone marrow. The conclusion of Alpen and Cranmore[6] and Erslev[7] that erythropoietin acts upon the stem cell was supported by morphological observations[1] since there is virtually no erythroid activity in the marrow or splenic red pulp of the polycythemic mouse, and 18 hours after a single injection of erythropoietin, proerythroblasts can be detected. On this basis we have undertaken a number of experiments exploring the regulatory mechanisms of the stem cell pool.[8,9] Recently, however, the simple concept that erythropoietin acts directly on the undifferentiated stem cell has been questioned by Bruce and McCulloch.[10] Their colony-forming cell, which must certainly be a stem cell, is not directly responsive to erythropoietin, since it is only after some days of an erythropoietic stimulus by hypoxia that the number of colony-forming cells decreases. Although it may be possible in the future to reconcile this observation with the original postulate that erythropoietin acts upon the stem cell, it is probably more fruitful to concede at this time that the colony-forming cell is the stem cell and can give rise to cells sufficiently primitive to escape morphological identification, but sufficiently specialized to have lost the ability to produce colonies. The original concept that the stem cell responds to erythropoietin, and, by differentiation, gives rise to the proerythroblast, has therefore been replaced by the postulate that an unknown stimulus regulates the differentiation of a colony-forming cell into a morphologically undifferentiated, erythropoietin-sensitive cell that has lost its ability to form colonies in the spleens of irradiated mice (Figure 1). The experiments described here are all directed at the nature and behavior of the erythropoietin-responsive cell (ERC).

*This report is taken from a paper that was presented at an International Symposium on Grafting Isologous Hemopoietic Cells, Paris, September 7-9, 1964.

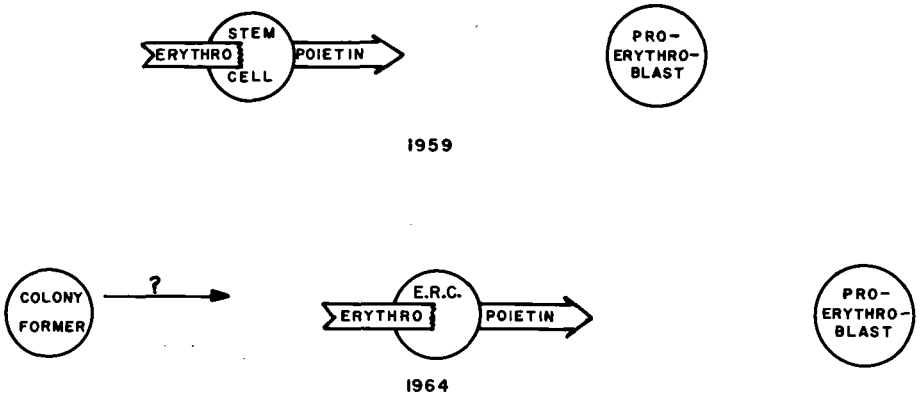

Figure 1

The following three series of investigations suggest that the ERC is multipotential, although admittedly this hypothesis cannot yet be considered as established beyond a doubt.

First, autoradiographic studies suggest that the cells potentially responsive to erythropoietin in the polycythemic Swiss mouse constitute a pool of cells rapidly turning over even when they are not actively producing erythrocytes. Spleens of polycythemic mice were used as a source of cells. A few deeply basophilic cells, the size of the large lymphocyte have been previously reported in spleen sections.[1] These cells increase strikingly in number as early as 15 to 18 hours after the subcutaneous administration of 6 units of erythropoietin. They are characterized by fine clumping of chromatin in the nucleus, intense basophilia of the cytoplasm, and a deep violet nuclear staining when Leishman-Giemsa stain is used: they will be referred to as dark blue cells (DBC). The time of appearance, size, and intense basophilia of these cells suggest that they are erythroblasts, but since the erythropoietin employed is not completely pure, the possibility that these distinctive DBC are produced in response to foreign protein cannot be excluded.

Experiments were then undertaken to see if DBC demonstrated avidity for radioiron. Mice were given 10 microcuries of Fe^{59} intraperitoneally 4 hours before sacrifice. Autoradiographs showed four types of cells concentrating Fe^{59} in the spleen. The first we believe to be an iron storage cell. It is a large, usually oblong cell with a uniform nucleus that takes only a moderately basophilic stain, and cytoplasm that shows almost no reaction with Leishman-Giemsa stain. It is the only cell in the spleen of polycythemic mice that shows radioiron concentration prior to erythropoietin administration. The second cell taking up Fe^{59} is the DBC. Many, but not all, of these cells seen in spleens of animals sacrificed 28 hours after erythropoietin and 4 hours after Fe^{59} administration show a striking concentration of the isotope over and around the cell. As early as 12 to 15 hours after erythropoietin there appears to be some concentration of Fe^{59} by many of the cells developing basophilia but not yet sufficiently advanced to permit their being classified as DBC. The few DBC present in the spleens of transfused mice given Fe^{59} without previous erythropoietin administration do not take up Fe^{59}.

The third type of cell which shows Fe^{59} incorporation is the newly formed erythrocyte. As

early as 28 hours after erythropoietin, polychromatophilic erythrocytes that concentrate Fe^{59} are seen. After 30 hours, well recognizable nucleated cells (normoblasts) of later stages of erythropoiesis can be identified as well. Although concentration of Fe^{59} by DBC does not in itself prove that DBC are erythroblasts, we believe this finding, considered in conjunction with appearance of these cells in blood forming tissue after administration of erythropoietically active material, is consistent with such a tentative opinion. The appearance of erythrocytes which take up Fe^{59} only 28 hours after erythropoietin administration is noteworthy because this precedes the appearance of reticulocytes in the peripheral blood and is so much earlier than the time of peak reticulocytosis[1] as to suggest that these erythrocytes may have been developed rapidly via the 2-dash pathway postulated by Lajtha and Oliver.[11]

Autoradiographic studies with tritiated thymidine give some insight into the nature of the ERC. A flash label was produced in vivo in transfused mice by a single intraperitoneal injection of tritiated thymidine (specific activity 1.9/curies/mm). Figure 2 shows a grain count distribution of nucleated erythrocytic precursors (DBC and normoblasts) from the spleens of such mice

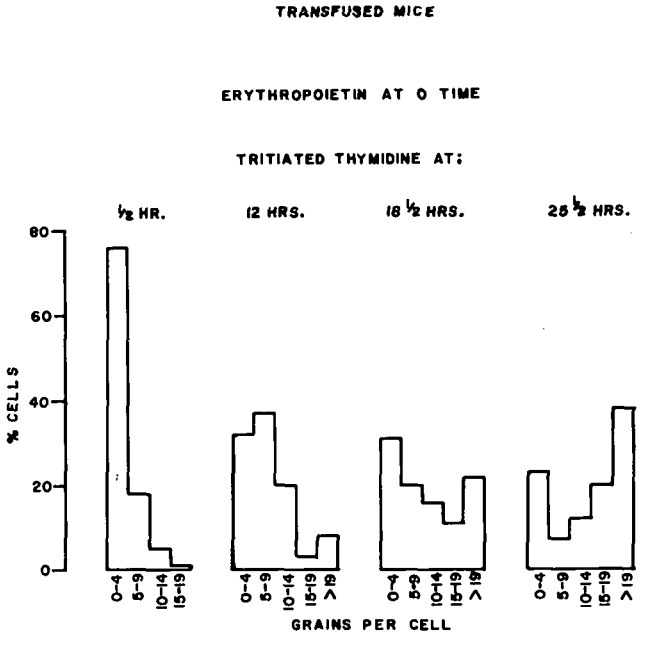

Figure 2

sacrificed 28 hours after injection of erythropoietin. Tritiated thymidine, 1 microcurie/gram body weight, was given to the animals at the times shown, and considerable tritiated thymidine uptake (and presumably therefore nucleic acid synthesis) is demonstrated in nucleated red cell precursors at various times after administration of the erythropoietic stimulus (Figure 2). Next, hypertransfused mice were given a flash label of tritiated thymidine (2 microcuries/gram body weight) one-half hour before administration of 20 units of erythropoietin. Approximately one-fourth of the DBC in spleens of animals sacrificed 20 hours after erythropoietin administration produced 20 or more grains per cell after 15 days of coated slide exposure (Figure 3). This number is probably falsely low, since it is reasonable to anticipate that at least one cell division will

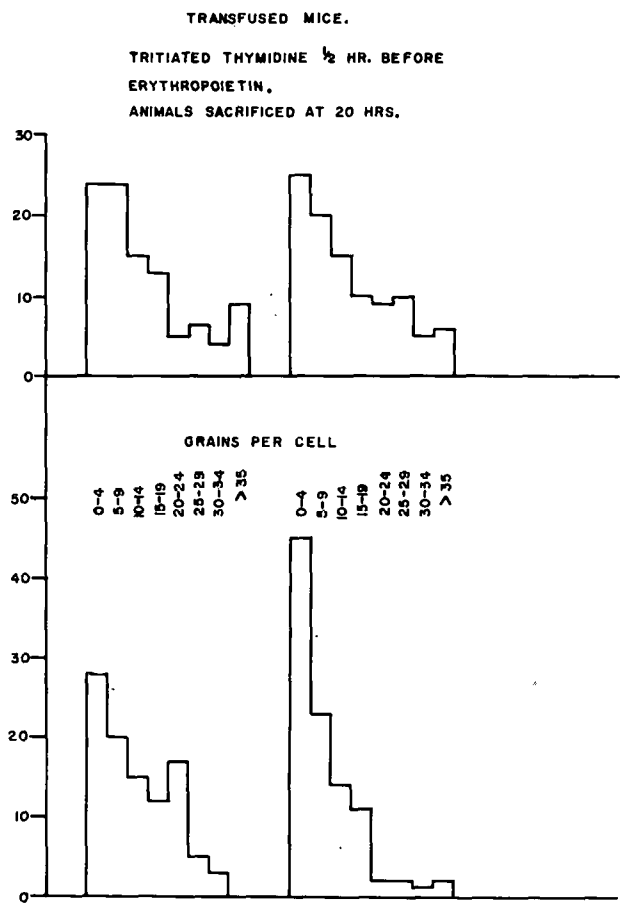

Figure 3

occur in a cell population first exposed to the stimulus for differentiation 20 hours previously. Reutilization by DBC of tritiated thymidine originally picked up by lymphocytes, granulocyte precursors, or other cells could theoretically account for these results, but this appears unlikely because of the short time between administration of thymidine and sacrifice. Although erythropoiesis is virtually absent in the transfused mouse,[1] we therefore conclude that many of the ERC are in cell cycle, as indicated by nucleic acid synthesis. This would be surprising if the ERC has no function other than self replication and production of erythrocytes, since when loss of cells by differentiation into the red cell series is eliminated there should be no need for self-replication. On the other hand, the result might be predicted if ERC is a multipotential cell, for although erythropoiesis is eliminated by plethora, turnover to compensate for loss of cells from this pool as a consequence of platelet, leukocyte, or other cell formation would be expected.

Second, evidence that the ERC is multipotential was uncovered during studies of the recovery of the erythropoietin sensitive cell after irradiation.[8,9] If total body irradiation is administered to polycythemic mice immediately before injection of a standard dose of erythropoietin, the decreased erythropoietic response is related to the dose of radiation.[12] This gives us a method for quantitation of the radiation damage to the erthropoietin sensitive cells. Figure 4

demonstrates radiation-induced impairment of response to 3 units of erythropoietin in plethoric mice of many strains. The simplest way of quantitating the erythropoietin response is to administer a tracer dose of Fe^{59} 48 hours after erythropoietin, and determine the percentage of the tracer dose that has been incorporated into newly formed red cells 72 hours later. When polycythemic CBA mice have been given 100 roentgens of radiation, and are challenged with a test

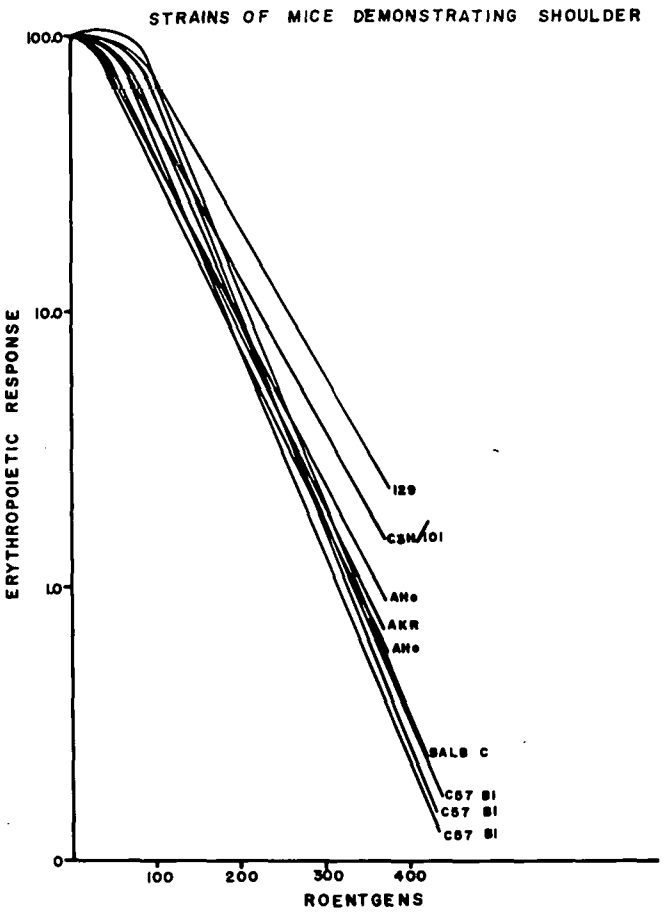

Figure 4

dose of 3 units of erythropoietin at different times after the irradiation, an interesting pattern emerges (Figure 5). Immediately after irradiation response is uniformly reduced to approximately 30 per cent of the response in the non-irradiated control. One day after irradiation, the response to erythropoietin is slightly lower: this finding has been reported elsewhere.[8,9] There is then a slow recovery followed by a plateau lasting for several days, and ultimately a rapid return to normal responsiveness. Figure 5 shows a recent experiment in which a prominent overshoot is observed. In the original experiments we failed to observe this overshoot,[9] but this rebound, originally reported by Alexanian, Porteous, and Lajtha[13] has been confirmed in our laboratory on several occasions. The important question here is why a compartment of cells capable of responding to erythropoietin, but not doing so in the polycythemic animal until the challenging dose of erythropoietin is administered, is able to recover from a radiation injury. Curtis and

Crowley[14] found that quiescent cells of the liver store radiation damage for long periods of time, and that manifestations of damage can be observed at a late date if partial hepatectomy is used to stimulate regeneration. Crooks et al.[15] found radiation damage latent in the thyroid gland for as long as 2 weeks between irradiation and the application of a stimulus for cellular proliferation. Similarly, we could have expected the continued presence of radiation damage in a compartment

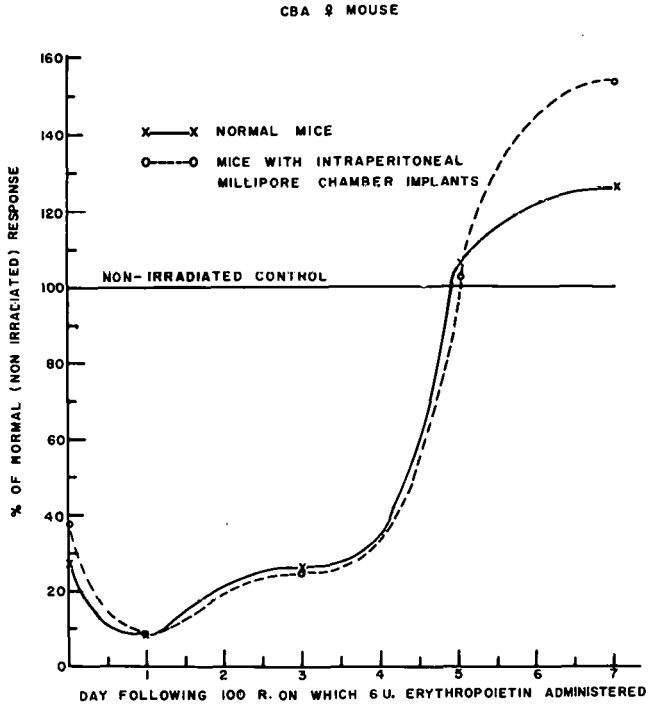

Figure 5

of primitive marrow cells which is arrested. The rapid recovery to normal and supranormal levels suggests that the compartment of cells responding to erythropoietin is also active in producing some other line of differentiated cells. In this, and in self-replication, damaged cells are culled out of the compartment.

Third, the idea that the ERC is multipotential arises from experiments on the mechanism by which the compartment of ERC tends to be maintained constant, a postulate which we believe to be a reasonable one. This experiment was designed before publication of Bruce and McCulloch's work,[11] when we had no reason to distinguish between the ERC and the stem cell. It was postulated that the number of stem cells was regulated by an inhibitor which was a by-product of stem cell metabolism. Were the number of stem cells to be reduced as a result of differentiation, the titer of inhibitor would fall, some stem cells would fail to be inhibited from going into cell cycle in preparation for division, and the pool, relieved of inhibition, would grow until a normal number of stem cells had been produced, when sufficient inhibitor would again be produced to hold the compartment constant. Such a concept was proposed by Weiss[16] to account for regeneration of parenchymal organs following partial ablation, and modified by Lajtha et al.[17] to account for a compartment of cells continuously undergoing attrition as a consequence of differentiation.

In an effort to test this hypothesis, the recovery of the ERC of plethoric CBA female mice given 100 roentgens and immediately thereafter subjected to implantation of a millipore filter chamber containing spleen slices was compared with the recovery of similar mice receiving empty chambers. In an effort to increase the number of stem cells in the slices, spleens of CBA mice which had been given 900 roentgens total body irradiation with shielding of the spleen, according to the techniques of Jacobson et al.,[18,19] were employed. As anticipated, recovery of the compartment of ERC was indeed delayed, as shown in Figure 6, but we suspect that this was not because of production of the hypothetical inhibitor by stem cells or ERC inside the chamber.

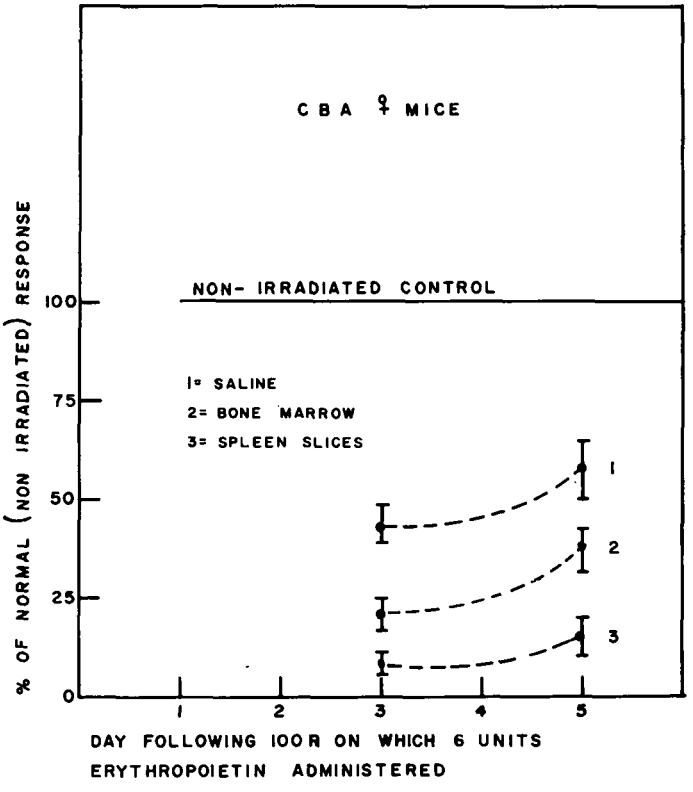

Figure 6

At autopsy a thick pseudocyst was found to encapsulate the chambers containing spleen slices, and the outer wall of such pockets was made up of a thick polymorphonuclear cuff (Figure 7, A-D). This interesting and unanticipated response was probably initiated by bacteria introduced within the chamber by infected spleen slices, since in sectioned chambers bacterial proliferation was seen within the chamber and migrating through the millipore filter walls. Moreover, the retardation of ERC recovery as a consequence of the presence of chambers containing spleen slices was eliminated when the irradiated recipient animals were given daily injections of penicillin and streptomycin. We therefore suggest that a reduced response to erythropoietin was obtained in such animals on the fifth day of irradiation because excessive demands were placed on the pool of ERC in the preceding five days, perhaps for leukocyte production. If so, the ERC must be multipotential; otherwise, the consistently retarded recovery in animals with chambers

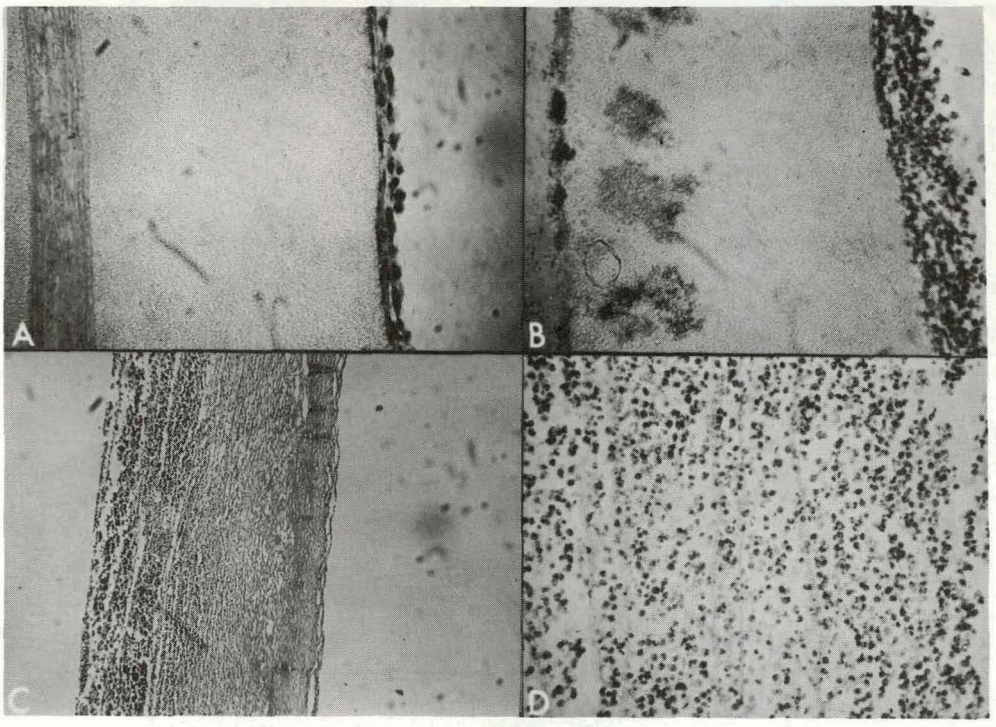

Figure 7

A. Section of control chamber (25X).
B. Section of chamber containing spleen slices (25X). Note polymorphonuclear leukocytic migration to peritoneal surface, and debris migrating through the filter.
C. Wall of pseudocyst, encapsulating chamber (4X).
D. Portion of wall of pseudocyst (25X).

containing infected spleen slices remains to be explained. This preliminary experiment is included in this discussion because: (1) the result is consistent with the concept we are presenting, (2) the pseudocysts were an unexpected observation which might prove to be of value in the investigation of experimentally induced inflammation and, (3) the concept underlying the original experimental design is worthy of consideration in this paper.

To summarize, three sets of findings suggest that the ERC's are multipotential: (1) their high rate of tritiated thymidine uptake in polycythemic mice, (2) their rapid recovery in irradiated polycythemic mice and, (3) their reduced responsiveness to erythropoietin in irradiated polycythemic mice when an inflammation is present in the abdominal cavity.

Implicit in our interpretation is the assumption that the primary loss of cells from the ERC compartment is attributable to differentiation, and that loss from cell death (excluding death of irradiated cells in mitosis) is a minor factor. Were a substantial rate of spontaneous cell death to be present in the ERC pool, this, rather than reduction of cell numbers by differentiation would, of course, constitute sufficient "force to drive the wheel" of compartment turnover in polycythemic mice.

The last experiments are concerned with following the course of a cluster of non-irradiated erythropoietin responsive cells when the remaining cells in the animal have been given a large dose of radiation. We hope to demonstrate further the existence of a delicately poised mechanism

of control of the number of ERC because we believe that acknowledgment of this mechanism will ultimately lead to its elucidation, and we believe that an understanding of it is important, both for experimental biologists and clinical hematologists. Spleens of CF No. 1 polycythemic mice were exteriorized and shielded, and the animals were then given 500 roentgens of total body irradiation. Immediately after irradiation, the spleens were returned to the abdominal cavities of the animals. Figure 8 shows the response to 3 units of erythropoietin, as a percentage of the response in non-irradiated animals, when the challenging dose of the erythropoietic stimulator was administered at different days after irradiation. Again an overshoot of 40 per cent of the normal

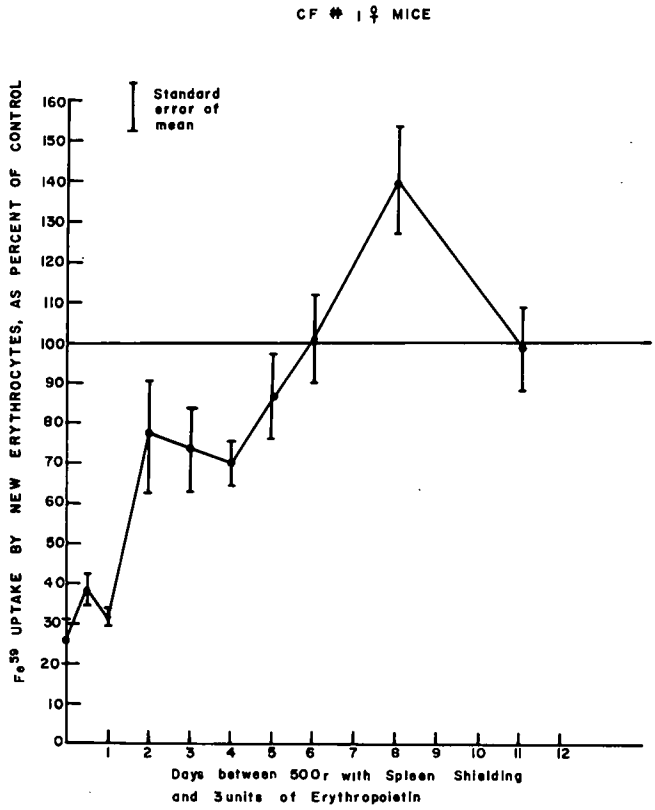

Figure 8

response can be observed on the eighth day, with a subsequent return to the normal on the eleventh day. This response curve is interpreted as reflecting the proliferation of the non-irradiated stem cells, since in our experience only a very small response to erythropoietin can be observed in the first week following 500 roentgens of total body irradiation to a non-spleen-shielded polycythemic mouse.

Although the previous experiment suggests that proliferation of splenic ERC follows irradiation, it was measuring over-all net responses to erythropoietin, and did not allow deductions as to the tissue responding, especially in animals challenged several days after irradiation. To find out whether response is confined in the spleen in the first few days following irradiation, the previous experiment was repeated, with radioiron given 36 hours after erythropoietin, and the animals were sacrificed 12 hours later. In this 12-hour period, the radioiron will be cleared

from the plasma, will not yet appear in the peripheral blood in newly formed erythrocytes to any appreciable degree, and will indicate by its organ distribution, compared with the distribution in polycythemic mice not given erythropoietin, where erythropoiesis is occurring.

Figure 9 shows that when erythropoietin is given to spleen-shielded animals immediately after irradiation, 6 per cent of the total dose of radioiron administered 36 hours later can be recovered from the spleen. When the administration of erythropoietin is delayed until 24 hours after irradiation, the uptake of radioiron has increased to approximately 11 per cent of the total

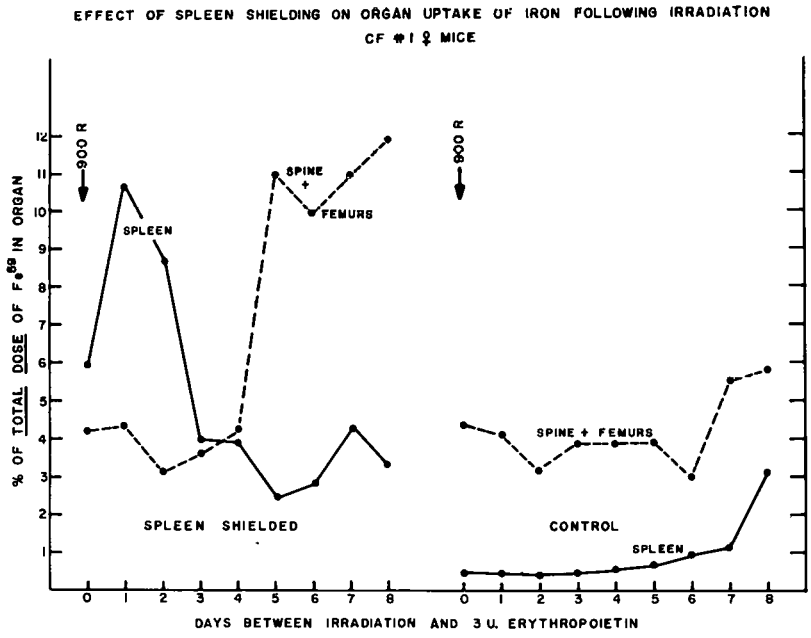

Figure 9

dose. This suggests that the number of erythropoietin responsive cells in the shielded spleen has doubled in the 24 hours following irradiation. Figure 8 would then suggest that there is then a decrease in the number of ERC in the spleen in the subsequent days, but that the number of ERC in the spine plus two femurs increases markedly after a delay of 4 days. We conclude that after proliferating in the spleen for 1 or 2 days most of the ERC leave the spleen to colonize the hemopoeitic system elsewhere. These two experiments taken together suggest a method of making inferences regarding the proliferation of erythropoietin responsive cells when a small cluster of them has been protected from damage, and the location of their progeny at various times after the initial damage.

In summary, we believe that these studies reflect the proliferative potential of a primitive cell of the mouse hemopoietic system. Erythropoietin has been used as a tool because it can be applied as a quantitative stimulus to this primitive compartment, the response to which can be measured with precision by radioiron.

These studies suggest that the erythropoietin responsive cell is in a dynamic pool even when erythropoiesis has been eliminated by hypertransfusion, and that the number of erythropoietin responsive cells tends to remain approximately constant. That is to say, a homeostatic mecha-

nism exists which tends to maintain this compartment. If most of the erythropoietin responsive cells in the animal have been damaged by radiation, forces come into play which tend to restore the cell number to normal. If a large dose of radiation destroys all but a cluster of protected cells, this cluster very promptly receives a message that initiates proliferation, and the cells quickly increase in number. When they are of splenic origin these cells tend to remain in the spleen for a short time, but after 24 hours their number in the spleen drops, and this fall precedes the appearance of erythropoietin responsive cells in the spine and femurs five days after the damage has been sustained.

At present, the mechanism by which the compartment of primitive cells can maintain itself in the face of continued loss due to differentiation, or can quickly recover when most of its cells have been damaged by irradiation or chemicals, should be of great interest to hematologists. A break-down of this regulatory mechanism, leading to continuous and autonomous proliferation of primitive cells may be relevant to the etiology and pathogenesis of an untreatable condition currently recognized by practicing hematologists with unfortunate frequency. Clearly many fruitful areas of exploration of primitive cell characteristics, behavior, and regulation remain for elucidation.

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QUANTITATION OF THE ERYTHROPOIETIC STIMULUS PRODUCED BY HYPOXIA IN THE PLETHORIC MOUSE*

By

C. W. Gurney, P. Munt, I. Brazell and D. Hofstra

The literature contains many references to the relationships between hypoxia and the production of the plethoric state,[1-5] the effects of altitude on blood volume,[6] reticulocyte response[7] and erythropoietin production.[8] The present study is concerned with the minimum period of hypoxia required to activate the organ of erythropoietin production in the mouse.

MATERIALS AND METHOD

Carworth Farms No. 1 virgin female mice, 7 to 9 weeks of age, were given 1 mg of Proferrin intraperitoneally. Plethora was then induced by residence in a pressure chamber as originally described by Cotes and Bangham[9] except for approximately 0.25 hour at ambient conditions 2 times weekly for cage maintenance and change of water. Details of chamber operation and results obtained have been reported elsewhere.[10] Following several days at ambient conditions, to permit maturation of erythroblasts already in cycle, a fresh wave of erythropoiesis was initiated by a single injection of erythropoietin,† a return to the chamber, or a combination of both. Forty-eight hours later, 0.5 μc of $Fe^{59}Cl_3$ (specific activity approximately 35 mc/mg of iron) in a volume of 0.2 ml was administered intravenously. In 2 experiments, the radioiron was brought to the desired volume by addition of a mixture of 1 part post-phlebotomy anemic mouse plasma to 4 parts of saline. In the remaining experiments, the radioiron was brought to volume with saline. Seventy-two hours later a measured volume of blood from the thoracic aorta was taken for scintillation well counting. From activity of a standard prepared at the time of radioiron injection, and assuming a blood volume of 7 per cent of the body weight for these animals, the percentage of the injected radioiron in newly formed peripheral red cells was determined.[10,12] It is assumed that this value is proportional to effective erythropoiesis, since both the radioiron incorporation of newly formed erythrocytes[12] and the reticulocyte response in plethoric mice[13] are functions of the amount of erythropoietin administered to these animals over a wide range of dose.

RESULTS

In the first experiment, mice were kept in hypoxia chambers for 28 days, at a pressure of 15 inches of mercury or approximately 0.5 atmosphere. After 4 days at ambient conditions, mice were returned to chambers for such variable periods that all the animals could then again be removed from the chambers simultaneously. Figure 1 shows the percentage of Fe^{59} incorporation

*The report is taken from a paper that appeared in Acta. Hemat. 33:246, 1965.

†Erythropoietin was obtained from the Hematology Study Section of the United States Public Health Service. It was derived from anemic sheep plasma and represented 720-fold purification of raw plasma. Its biological activity is expressed in units as defined by Goldwasser and White.[11]

in individual animals after zero time, 5 minutes, 30 minutes, 1 hour, 4 hours, or 8 hours in the chamber or after intravenous administration of 1 unit of erythropoietin. A clear erythropoietic effect can be noted after 4 hours of hypoxia, and a greater erythropoietic response at 8 hours.

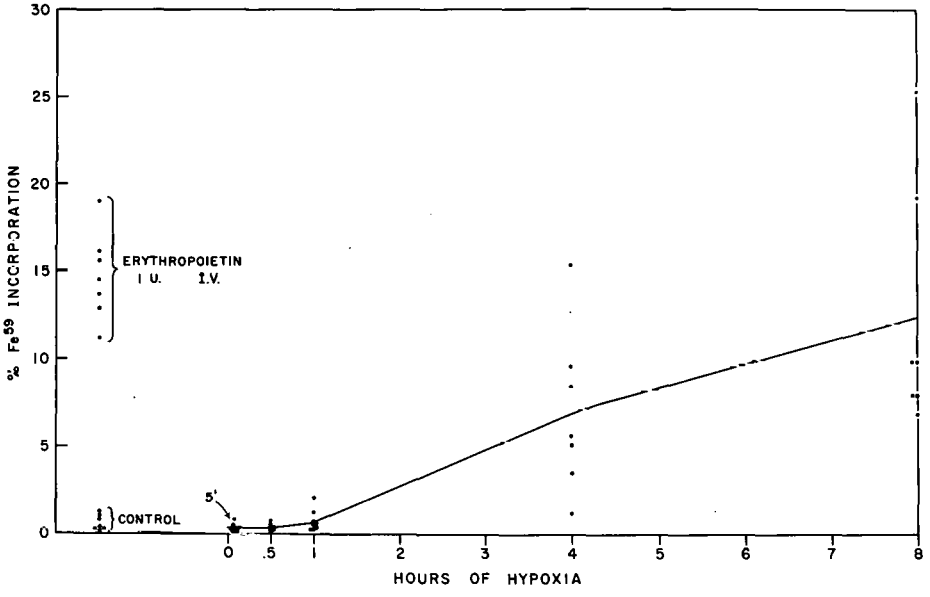

Figure 1. Response to hypoxia and erythropoietin in the hypoxia-induced plethoric mouse.

In the second experiment, after 26 days of hypoxia, mice were returned to the chamber for 0, 1, 2, 4, 8, or 24 hours. Again the return to different chambers was scheduled so that all animals would be finally removed at 4 days after their initial removal. One group of mice was then removed from the chamber, given 3 units of erythropoietin subcutaneously, and immediately returned to the chamber for an additional 4 hours. A second group of mice received radioiron in saline instead of in an anemic mouse plasma-saline solution. All the other mice in this experiment received radioiron in anemic mouse plasma-saline solution. Minimal stimulation of erythropoiesis was observed in 5 of 9 mice following exposure to 0.5 atmosphere for 1 hour, and, as seen in Figure 2, erythropoiesis increased with longer periods of hypoxia up to 24 hours. Erythropoiesis was more than twice as great after 2 consecutive periods of 4 hours of hypoxia separated by injection of 3 units of erythropoietin, than after a continuous 8-hour period of hypoxia. There was no significant difference in radioiron uptake when radioiron was given in anemic mouse plasma-saline solution.

In the third experiment, after 22 days at 0.5 atmosphere and varying times at ambient conditions, some animals were returned to the chamber for 6 hours at 0.5 atmosphere, others were given 2 units of erythropoietin subcutaneously. Groups of untreated plethoric animals were used as controls. All radioiron was given in an anemic plasma-saline mixture. Figure 3 demonstrates the response to a standard hypoxic stimulus or to a standard dose of erythropoietin administered on the second, fifth, or eighth day at ambient conditions following removal from the chamber. Between the second and eighth days, erythropoietic activity decreased slightly in the control animals, from an average value of 3.5 per cent iron incorporation to 0.4 per cent. There was no

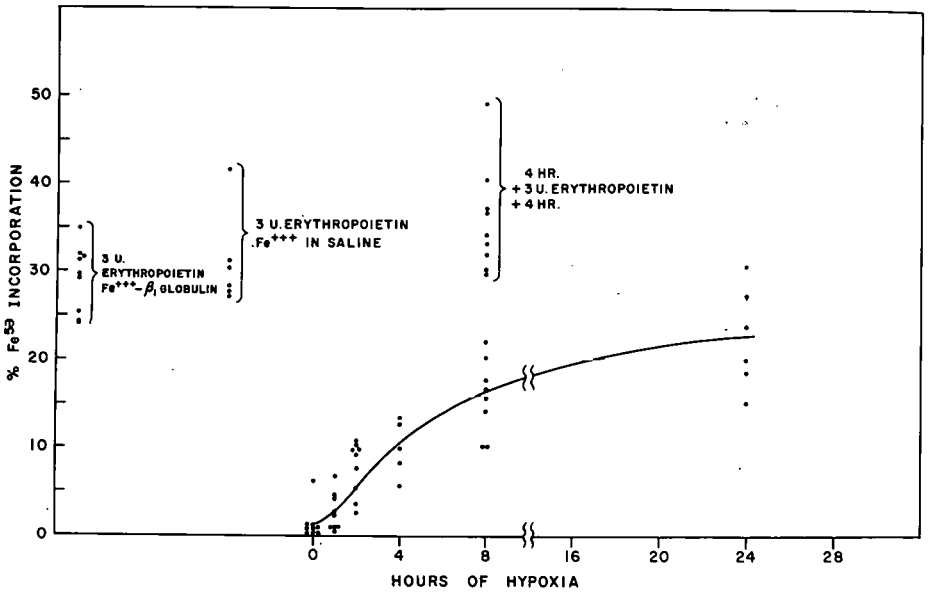

Figure 2. Response to hypoxia and erythropoietin in the hypoxia-induced plethoric mouse.

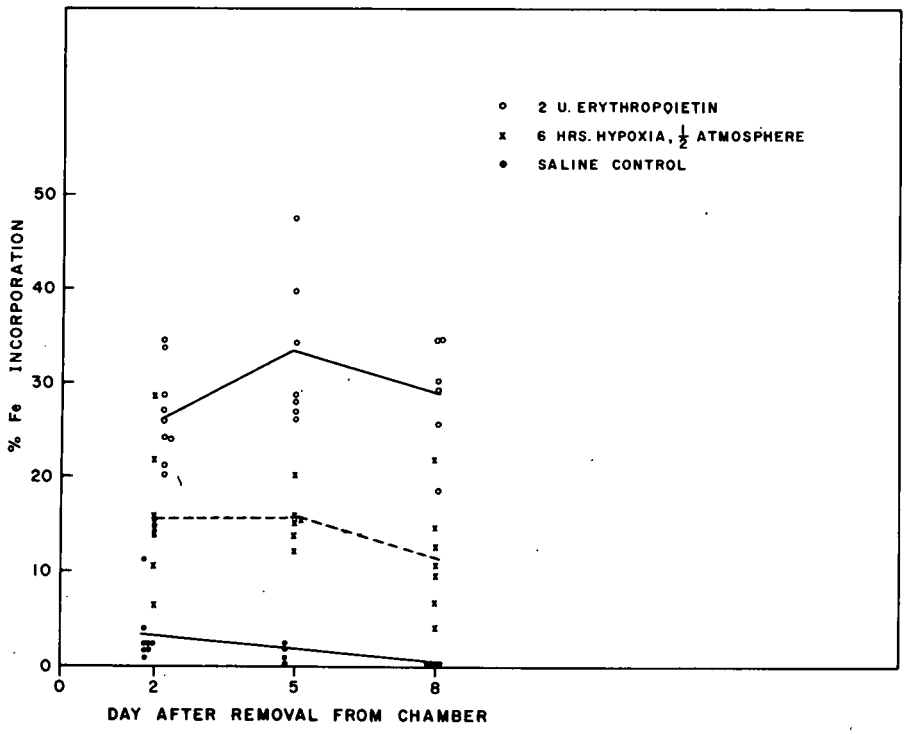

Figure 3. Response to hypoxia or erythropoietin in the hypoxia-induced plethoric mouse at various times after cessation of prolonged hypoxia.

clear difference of response to a fixed 2 unit dose of erythropoietin, or to 6 hours at 0.5 atmosphere, during this interval.

A series of experiments was designed to explore more intense hypoxia. After 22 days of exposure to 0.5 atmosphere, and 5 days of ambient conditions, groups of 13 plethoric mice were returned to chambers for 6 hours at different pressures. Ten non-acclimatized control mice of the same age accompanied each group of plethoric mice in order to ascertain the degree to which acclimatization enhanced survival. Mice previously acclimatized at 0.5 atmosphere can survive for 6 hours at 0.33 atmosphere, but not at 0.25 atmosphere, while 4 of 10 non-acclimatized mice failed to survive 0.33 atmosphere. That the erythropoietic stimulus produced by 6 hours of hypoxia is related to the severity of the hypoxia is demonstrated by the radioiron incorporation, which averaged 9.5 per cent after exposure to 0.5 atmosphere and 20.9 per cent after exposure to 0.33 atmosphere. In this and the following experiments radioiron was administered in saline, instead of in anemic plasma-saline mixture.

We next examined the erythropoietic response following return to an environment of pronounced pressure reduction for a shorter interval of time. After 24 days at 0.5 atmosphere and 4 days at ambient conditions, 3 groups of 10 mice were returned to chambers for 1 hour at pressures indicated in Figure 4. Eight control animals were not returned to the chamber. Some animals showed an erythropoietic response and others failed to respond to 1 hour's exposure at 0.5 atmosphere, but all animals exposed for 1 hour to environments of 0.4 or 0.33 atmospheres showed a clear erythropoietic response. Although individual responses within each group varied widely, the average erythropoietic response increased rapidly as the pressure decreased from 0.5 to 0.33 atmospheres.

In the final experiment, after 33 days exposure to 0.5 atmosphere and 4 days at ambient conditions, groups of mice were returned to the chamber at either 0.25 atmospheres or 0.27 atmospheres pressure. Non-acclimatized mice of the same age were also subjected to the shorter periods of exposure, to assess survival enhancement by acclimatization. A previous prolonged exposure to hypoxic conditions was associated with a substantial degree of protection against the lethal effect of short periods of intense hypoxia. At 0.25 or 0.27 atmospheres, exposure for 20 minutes or less resulted in very slight stimulation in a few animals within the group, and since many of the animals failed to respond, standard deviations of the mean of these groups were high. At these pressures, 30 minutes was sufficient to produce a clear erythropoietic stimulus, and the response was greater at 45 minutes.

DISCUSSION

Normal mice approach a new hematological equilibrium after approximately 3 weeks at a pressure of 0.5 atmosphere, a condition simulating an altitude of 18,000 feet.[10,14] Longer periods of hypoxia do not further increase the hematocrit of blood from the tail vein, although the red cell mass and the plasma volume may continue to increase after the hemoglobin concentration has stabilized in a hypoxic environment.[15,16] It seems likely that once the plethoric state is established, an environmental pressure of 0.5 atmospheres no longer produces the striking hypoxic stress exerted by exposure without previous acclimatization. Following return to ambient conditions, the abundance of blood relative to need is such that erythropoiesis is reduced markedly.

We presume, but cannot yet prove, that the erythropoietic stimulus resulting from a brief

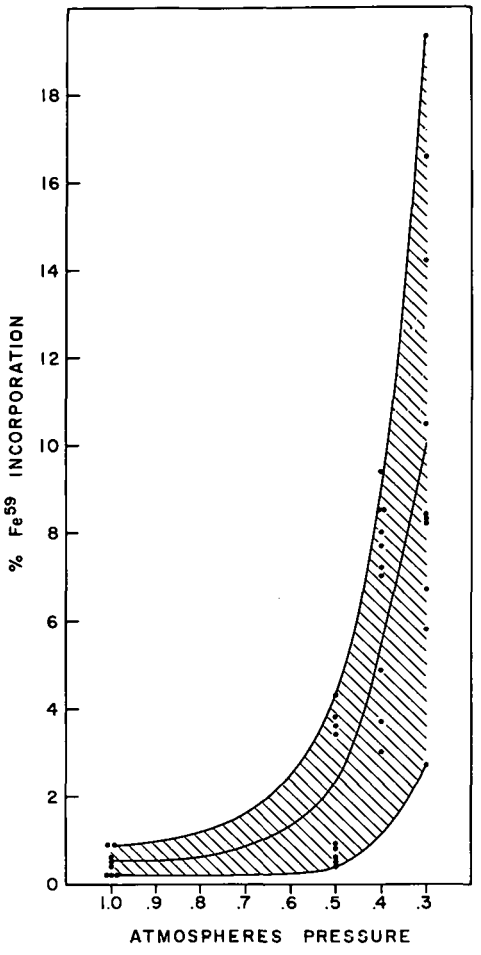

Figure 4. Response to variable degrees of hypoxia of one hour duration in the hypoxia-induced plethoric mouse.

return to 0.5 atmospheres is entirely dependent upon endogenous erythropoietin elaboration, although alternative mechanisms might be implicated. As yet it has not been shown that erythropoietin is always responsible for activation of erythropoiesis. Stohlman[3] does not agree that erythropoietin is the regulator of normal erythropoiesis, and an alternative mechanism that operates under normal circumstances might also account for erythropoiesis when animals adapted to 0.5 atmosphere pressure are returned to these "normal" conditions. However, such an alternative mechanism has not yet been demonstrated and the simplest postulation at this time is therefore that the erythropoietic activity observed in the animals returned to the hypoxia chamber is a consequence of the endogenous elaboration of erythropoietin.

In order to elicit demonstrable erythropoiesis in animals presumably elaborating no erythropoietin, it appears that the hypoxic stimulus must be operating for approximately 1 hour, when it is of the same intensity as that to which the animals were previously exposed. The stimulation produced by 1 hour at 0.3 atmosphere, and by shorter intervals with more severe hypoxia indicate that even shorter periods are sufficient to initiate an erythropoietic stimulus, and presumably for the elaboration of erythropoietin. Some animals show a slight response to as little as 15

minutes exposure to severe hypoxia.

A sudden return to 0.5 atmosphere undoubtedly constitutes an erythropoietic stimulus, but it appears unlikely that it is comparable to that experienced by animals not previously conditioned. The stimulus experienced by previously adapted mice in 24 hours is somewhat less than that produced by a single subcutaneous injection of 3 units of erythropoietin. A dose response curve should make it possible to estimate the erythropoietic stimulus for any altitude and any period sufficiently short to approximate the time over which a single injection is active.

A number of ancillary questions have also been investigated during the present study. First, the necessity for injecting radioiron bound to mouse iron-binding protein was considered. By previous bleeding of donor mice, plasma with an unsaturated iron binding capacity of 250 γ per cent was obtained, and a small amount of this plasma was sufficient to insure complete binding of radioiron of high specific activity. This precaution appears to have been unnecessary since we found the average radioiron incorporation following 3 units of erythropoietin was 29.7 per cent of the half μc injected when given with mouse plasma, and 31.2 per cent when given as $Fe^{59}Cl_3$ in saline. We therefore abandoned anemic plasma-saline mixture in favor of normal saline for diluting radioactive ferric chloride.

The degree to which the bone marrow might become refractory to erythropoietin, and the organ of erythropoietin elaboration might become refractory to a hypoxic stimulus after a prolonged period of decreased activity are of some importance in determining the optimal time for administration of the erythropoietic factor, and may also be relevant to mechanisms of production of erythropoietin. We found little if any change either in elaboration of erythropoietic factor or in response to exogenous erythropoietin over the 6-day period. A simple demonstration of erythropoietin elaboration or marrow sensitivity in the first 48 hours following cessation of the prolonged hypoxic stimulus is impossible only because maturation of erythroblasts continues for several days following removal from the chamber, and high baseline results are therefore obtained in unstimulated animals. Between the fourteenth and seventeenth day after removal from the chambers the hematocrit falls to less than 55 per cent, below which endogenous erythropoiesis may be resumed.[14] Since the optimal assay of erythropoietin or the erythropoietic effect of a short period of hypoxia requires 5 days, it is not practical to extend the study beyond the eighth day after removal from the chambers unless the conditions of preparing the plethoric state are altered or fortified by blood transfusion.

Finally, if the erythropoietic response in plethoric animals returned to a hypoxic environment is the consequence of elaboration of erythropoietin, it is of interest to consider whether this response is limited by the amount of erythropoietin elaborated, or by the sensitivity of the bone marrow under hypoxia. While there is as yet no definitive answer to this question, the response in animals receiving 3 units of erythropoietin preceded and followed by 4 hours of hypoxia is strikingly greater than in animals exposed for 8 hours to hypoxia, and is slightly greater than the response observed in animals receiving 3 units of erythropoietin alone, suggesting that at least in a mildly hypoxic state, the bone marrow responds to erythropoietin, in proportion to the amount of erythropoietin elaborated. Since the precise duration of activity of intravenously administered erythropoietin is not known, the summation of erythropoietin and hypoxia must be observed at longer intervals to exclude the possibility that the injected erythropoietic factor persists in the plasma or the bone marrow and exerts its primary stimulatory effect after removal from the chamber.

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IMPAIRED DELAYED RESPONSE FROM THALAMIC LESIONS IN MONKEYS*

By

S. Schulman†

The relevance of the thalamus to intelligent behavior, which is suggested by its cortical connections, and by a few impressive, if isolated instances of selective thalamic disease in man,[1-3] has been difficult to verify experimentally. While deficits in problem solving have been observed in rats[4-6] and cats[7,8] with thalamic lesions, similar studies with monkeys have yielded negative results. Walker[9] and Peters, Rosvold and Mirsky[10] found no impairment in delayed response performance in monkeys with bilateral lesions of the dorsomedial nucleus, and Chow,[11] likewise, was unable to induce defects in delayed response by combined lesions in the pulvinar and dorsomedial nuclei.

The extent of the lesions in Walker's experiments was not described in detail, but it is clear that the dorsomedial nuclei were not completely destroyed in any of the animals described by Chow, or by Peters, Rosvold and Mirsky. These investigations therefore are not entirely conclusive, especially in view of the equipotentiality that so often becomes apparent in ablation experiments involving higher levels of the nervous system. In this connection, the more recent study of Brierley and Beck[12] is of interest. These authors found distinctive changes in temperament in monkeys with almost complete bilateral destruction of the dorsomedial nuclei. Their animals showed a great decrease in fear of humans, and gave a general impression of "fatuous equanimity."

The experiments to be presented here were undertaken to determine whether the lack of effect of dorsomedial lesions in monkeys on delayed response, which has heretofore been found, may be a consequence of preservation of portions of the nucleus. The lesions were made by implanting radioactive sources in the thalamus, rather than by the customary electrolytic technique, because of the difficulty always encountered with the latter method in making large lesions which conform to the spheroidal configuration of the dorsomedial nucleus.

The animals were trained in a conditional visual discrimination problem, in addition to delayed response, in order to assess the degree of specificity which might attach to any defects produced in delayed response. Monkeys no. 1, 3 and 6, were also trained in a tool-using problem, with results to be described in a separate report.

METHODS

Subjects. The subjects of these experiments were 9 test-naive rhesus monkeys (Macaca mulatta). Six males (no. 1, 3, 6, 11, 22 and 24) and 3 females (no. 7, 21 and 26). Body weights at the time of surgery ranged from 3 to 8 kg.

Technique of producing lesions. The thalamic lesions were made by the permanent implantation of pellets of solid yttrium oxide, containing yttrium^{90}, a beta-emitting radioactive isotope

*This report is taken from a paper that appeared in Arch. Neurol., 11:477, 1964.

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with a half-life of 64 hours. The finite and relatively limited tissue penetration of beta particles makes this type of radiation well-suited for the production of circumscribed lesions in the brain. Yttrium^{90} was selected because of its convenient half-life and dose-rate, and the insolubility of yttrium oxide in body fluids. The size of the lesion resulting from a single pellet is related quite consistently to the activity of the pellet. Figure 1, which illustrates this relationship, was constructed from data reported by Harper, Lathrop and Kennedy,[13] and was used as a guide in

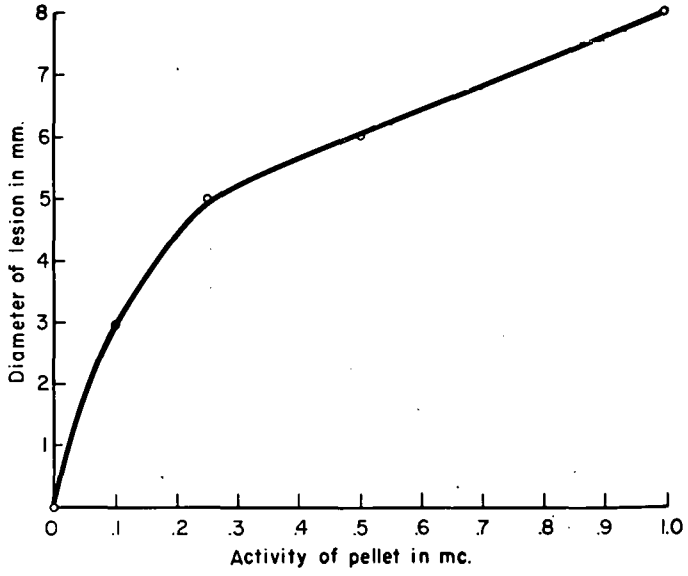

Figure 1. Relation between beta-ray dosage and size of lesion from data of Harper, Lathrop and Kennedy[13].

choosing appropriate dosages for various sites in the thalamus. The pellets are made by tamping down a small quantity of yttrium oxide powder in the lumen of a 19 gage needle, and ejecting the resulting cylinder of powder into a recrystallized alumina crucible, which is then placed in an induction furnace at 1650 degrees C for 2 hours. This baking produces cylindrical pellets of a hard, ceramic-like consistency, approximately 0.8 mm in length and 0.6 mm in diameter. Irradiation of the pellets was carried out at the Argonne National Laboratory, Argonne, Illinois. On delivery, each pellet was assayed. The time required for decay to the desired activity was calculated, and the pellet implanted in the animal after this time interval had elapsed.

The surgical procedures were carried out under aseptic conditions in the following manner: The animal was fixed in a Lab Tronics stereotaxic instrument under light intravenous pentobarbital anesthesia. The scalp was incised, and a small opening was made in the skull with a dental drill. The dura was opened sufficiently to admit an 18 gage needle freely. An 18 gage needle, with fitted stylet in place, was held and oriented by an electrode carrier and inserted into the brain to a level 1 mm below the desired horizontal plane. After an interval of 5 minutes, the needle was elevated 1 mm and the stylet was withdrawn. The radioactive pellet was then dropped into the hub of the needle from the lumen of a lead-shielded 16 gage needle, and guided gently down the shaft to the tip of the 18 gage needle in the brain by means of the stylet. The latter was

again withdrawn, and another stylet, with a smoothly rounded tip, and 5 mm longer than the needle itself, was inserted into the lumen and lowered with a previously calibrated electrode carrier until its tip projected 0.5 mm beyond the tip of the needle. The long stylet was held in place while the needle was withdrawn a distance of 3 or 4 mm, and then both were withdrawn completely. Unless these steps are followed in detail, the pellet is likely to adhere to the tip of the needle, and to be deposited considerably higher than is desired.

In 3 of the subjects (monkeys no. 1, 7 and 11), a single pellet with an activity of from 0.8-1.2 mc was placed in each thalamus. In monkeys no. 3 and 6, 2 pellets with activities of from 0.3 to 0.8 mc were placed in each thalamus. Three pellets with activities between 0.3 and 0.5 mc were placed in each thalamus in monkeys no. 21,22,24, and 26. The pellets were implanted one at a time, at intervals of 1 to 3 weeks. The use of more than one pellet of relatively low activity, and placed at different sites, rather than single pellets of high activity, permitted greater flexibility in determining the form and extent of the areas of necrosis in the thalamus. Although this procedure is laborious and time-consuming, earlier attempts to deposit more than one pellet simultaneously resulted in fatalities from extreme swelling of the thalamus and closure of the third ventricle. It is almost unlikely, however, that even temporary obstruction occurred in any of the animals of this series following the implantation of one pellet at a time, in view of the very mild clinical manifestations which were observed during the first post operative week.

The atlas of Olszewski[14] was used in selecting the stereotaxic coordinates. Prior to the implantation of the first pellet lateral x-ray views of the skull were made under light pentobarbital anesthesia, with small metallic spheres placed in the external auditory meati, to determine the correction factor for frontal plane numbers according to the method suggested by Olszewski.

Reconstruction of lesions. After postoperative observations were completed, the animals were sacrificed with an overdose of pentobarbital, and the brains were perfused with saline followed by 10 per cent formalin via the left ventricle of the heart. The brains were removed, immersed in 10 per cent formalin for 7 days, embedded in celloidin, and sectioned serially at 18 microns in the frontal plane. Pairs of sections at intervals of 20 throughout the thalamus, and at intervals of 10 throughout the rostro-caudal extent of the lesions, were stained by the Klüver method for myelin sheaths, and with cresyl violet. The areas of destruction were outlined on reproductions of Olszewski's diagrams at intervals of 0.6 mm and the lesions were measured with a planimeter. Curves were constructed by plotting the areas against their frontal plane numbers. The volume of tissue destroyed in each animal was approximated as described by Dornfeld, Slater and Scheffé.[15] The volumes of the entire normal thalamus and of the intact dorsal medial nucleus were measured in similar fashion so as to permit a reasonably precise estimate of the per cent destruction of this nucleus and the thalamus in each animal (Table 1).

Representative areas of all portions of the cerebral cortex of each monkey were also examined microscopically and compared with normal controls. Cresyl violet and hematoxylin and eosin preparations were made from histological sections of the eyes of 3 of the subjects (monkeys no. 21, 24 and 26) and 1 normal control.

Neurological examination. Neurological examinations were carried out routinely preoperatively and 1 week following each operation, and several times thereafter. Eye movements, pupillary reactions to light and on convergence, muscle tone, deep reflexes, pain reactions to an alligator clip, and recognition of edible objects placed in the hands outside the field of vision were examined with the animals restrained in a primate chair. Visual fields were examined by bring-

Table 1

PER CENT DESTRUCTION OF DORSOMEDIAL NUCLEUS AND THALAMUS*

	Dorsomedial Nucleus			Thalamus		
Monkey	Left	Right	Total	Left	Right	Total
24	100	100	100	33	33	33
26	100	100	100	43	46	44
6	100	98	99	41	31	36
21	98	90	94	40	39	40
22	97	76	86	39	29	34
11	74	93	84	16	30	23
7	80	77	78	35	31	33
1	61	88	74	17	22	20
3	48	50	49	16	20	18

*The figures for per cent destruction are based on planimetric measurements of the areas of normal structures and of the lesions drawn on standard diagrams of the thalamus. See text under Reconstruction of lesions. The subjects are listed in the same order here as in Figures 3, 6 and 7, and Table 2.

ing food into the periphery of the fields with the monkey's gaze fixed on another piece of food held directly in front of his face, and also by giving the animal access to a row of banana sections on a table surface extending into the periphery of the right and left fields. Taste sensation was examined by presenting sections of banana soaked in a saturated solution of quinine hydrochloride.

Behavior tests. Each animal was given pre- and postoperative training in spatial delayed response and in a conditional visual discrimination problem. The tests were given in the order mentioned both pre- and postoperatively to all of the subjects except monkeys no. 21 and 22, who were given the visual discrimination problem first, followed by delayed response. The non-correction technique was used in both tests. Reward for correct responses was always a small slice of banana. The animals were fed once daily immediately after testing. The diet consisted of commercial biscuits daily (Purina Monkey Chow), an apple or orange 3 times weekly, and a hard-boiled egg and supplementary vitamins once weekly. The amount of food was limited to that required to maintain approximately 80 per cent of body weight on an ad lib diet.

The delayed response tests were conducted with the animal in a metal cage consisting of delay and response compartments, each 20" by 20" by 20", separated by 2 vertically sliding metal screens, one opaque and the other containing a grille made up of 4 vertical openings 2" wide. The choice points were 10" apart and covered by identical aluminum cups 3" in height and 2-1/2" in diameter. The tops and all sides of both compartments were made of 1/2" metal mesh, permitting observation of the subject during the delay interval, as well as at the time of response. After the subjects were habituated to the cage and had learned to displace the cups to obtain the reward, training was begun in daily sessions of 20 trials each, with inter-trial intervals of approximately 10 seconds. The balanced left-right sequences of Gellermann were used to determine the order of the positive sides. The initial pre- and postoperative sessions for all but 2 of

the subjects were conducted under "closed, minimum-delay" conditions, in which the procedure for each trial was as follows: With the monkey observing from the delay compartment through the lowered grille screen, the reward was placed on the positive side and covered with a cup, the negative side was covered with a cup, the opaque screen was lowered, and then both the opaque and grille screens were immediately and simultaneously elevated to permit the subject to enter the response compartment and displace one of the cups. Selection of the correct cup was rewarded by the food which it concealed. Incorrect responses were penalized only by non-reward. Following incorrect responses, the animals were prevented from displacing the correct cup by prodding them back into the delay compartment with a metal rod. All of them quickly learned to return spontaneously to the delay compartment after incorrect responses, so that the physical restraint was no longer necessary after the first few preoperative sessions. When the criterion of 80 per cent correct responses in 100 consecutive trials was achieved under "closed, minimum-delay" conditions, a delay of 5 seconds was introduced between the lowering of the opaque screen and the raising of both. Subsequently, the delay intervals were increased in steps of 5 seconds each when the criterion of 80 per cent correct responses in 100 consecutive trials was reached at each delay. In the case of monkeys no. 21 and 22, pre- and postoperative testing in delayed response was begun under "open, minimum-delay" conditions, in which the opaque screen was not lowered. Instead, the grille screen was immediately raised after the stimulus cups were set up. When criterion under these conditions was reached, "closed, minimum-delay" trials were given, and the subsequent procedure was the same as for all of the other subjects. The scores of monkey no. 22 in the pre- and postoperative open, minimum-delay trials were discarded, since they showed nothing but the expected postoperative savings in an animal without impairment in delayed response. They were retained in monkey no. 21, however, because of the exceptional difficulty exhibited by this subject in the postoperative open, minimum-delay trials. In 3 subjects with postoperative impairment in delayed response under standard conditions (monkeys no. 6, 11 and 26), testing was repeated using a pre-delay exposure technique, in which, on each trial, the positive side was baited and the reward then left exposed for 15 seconds before the cups were inverted over the choice points and the opaque screen lowered.

The conditional visual discrimination test was conducted with the subjects in a 24" by 24" by 24" cage on the surface of a table. The cage was closed on all sides except the front, which was made up of vertical bars spaced 1-1/2" apart, and fitted with an opaque screen which could be raised and lowered. Stimulus plates were placed over 2 food wells spaced 11" apart center-to-center in a wooden stimulus tray adjacent to the front of the cage. With the screen lowered, the positive well was baited with a section of banana and covered with the positive stimulus plate, and the other covered with the negative stimulus plate. The screen was then raised to permit the subject to displace one of the stimulus plates. Left and right sides were baited according to Gellermann's sequences. The discriminanda were black and white squares, the size of the squares serving as the conditional cue. The small squares were 1-1/4", the large 2-7/16". Training was given in 4 steps to all the subjects, but the schedule used for monkeys no. 21, 22, 24 and 26 differed slightly from that for monkeys no. 1, 3, 6, 7 and 11. The preoperative scores for the two groups of animals showed no significant differences, however, and the two methods may be regarded as equivalent. With subjects 21, 22, 24 and 26, training was carried out in daily sessions of 25 trials to a criterion of 90 per cent correct in 50 consecutive trials in the following 4 steps:

1. Small black (+) vs. small white (-).

2. Large white (+) vs. large black (-).
3. Small black (+) vs. small white (-) and large white (+) vs. large black (-) in alternating blocks of 5 trials each.
4. Small black (+) vs. small white (-) and large white (+) vs. large black (-) presented in balanced, random order.

With subjects 1, 3, 6, 7 and 11, steps 1, 2 and 4 were given in daily sessions of 30 trials each to a criterion of 90 per cent correct in 30 consecutive trials. Step 3 was given in sessions of 40 trials each, the large squares being presented in one block of 20 trials, and the small squares in another block of 20 trials. Criterion in this step was 90 per cent correct in 40 consecutive trials.

All animals were given a generalization test following the training in square patterns, both pre- and postoperatively. This consisted of 4 sessions of 30 trials each. In the first 10 trials of each session, the small and large squares were presented in randomized order as in step 4 above. In the second block of 20 trials of each session, a new geometrical pattern was presented in random order. These were rectangles, diamonds, circles and crosses. The largest diameter of the new patterns corresponded to the size of the squares. As in the testing with square patterns, the small black and the large white patterns were positive, but in the generalization trials, both correct and incorrect responses were rewarded.

RESULTS

Thalamic Lesions

The lesions produced by pellets of yttrium^{90} and other small beta-ray sources have been described by Harper, Rasmussen, Kennedy and Gerol.[16] With yttrium sources the maximum degree of acute swelling occurs between 48 hours and 8 or 10 days following implantation. The size of the definitive zone of necrosis is probably established and stable before the end of 2 weeks. Within a few months after implantation the lesion consists of a roughly spherical region of complete necrosis surrounded by a narrow, sharply defined border of macrophages and fibrous tissue (Fig. 2). The diagrammatic appearance of such lesions, however, is transformed with the passage of time by resorption of the debris, shrinkage of the cystic cavity and a certain degree of puckering of the surrounding tissue. The neurons in the immediate vicinity of old lesions retain their normal appearance, but exhibit a characteristic change in orientation, their long axes coming to lie along lines radiating from the shrunken lesion as a center. Such distortion was present in all animals of the present series, since they were sacrificed between 1 and 2 years following surgery.

The per cent destruction of the dorsomedial nucleus and thalamus in each animal is given in Table 1, and the lesions are shown in diagrammatic form in Figure 3.

The lesions in monkey 21 are of special interest in view of her good postoperative performance in the delayed response test in spite of very extensive destruction of the dorsomedial nuclei (Figure 3D). The lesions extended beyond the borders of the dorsal medial nuclei to involve other thalamic structures to a variable degree in all of the animals. Extension of the lesions beyond the borders of the thalamus dorsalis proper also occurred in all of the animals, but only to a minimal degree.

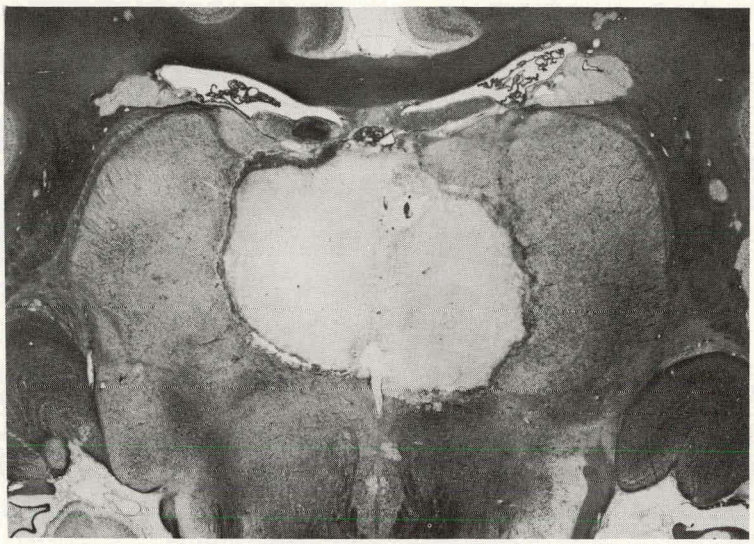

Figure 2. Yttrium^{90} lesions of the dorsomedial nuclei in an untrained monkey sacrificed 2 months after operation. Note ovoid shape and narrow, sharply-defined borders. Luxol fast blue and cresyl<violet.

Changes in General Behavior

With pellets having an activity of 0.5 mc or more, there were distinct, but mild and transient, acute effects appearing on the third postoperative day, and subsiding after the fifth or sixth day. These consisted of drowsiness, a disinclination to move about, and anorexia. In addition, a relative neglect of objects in the contralateral visual field was often observed, similar to that described by Kennard[17] and Clark and Lashley[18] after lesions of the frontal cortex. This rarely persisted for more than 10 days, and never longer than 3 weeks.

Drowsiness as an effect of these thalamic lesions was confined to the first postoperative week, and seems best attributed to transitory edema involving more widespread areas in the diencephalon than those which were destroyed. A single partial exception to this statement is monkey no. 24 who tended to become drowsy and fall asleep when restrained in the primate chair and left unstimulated for a few minutes.

All of the subjects exhibited additional alterations in general behavior, which did not persist in prominent form beyond the second or third month after surgery. They were not seen until after the second pellet was implanted, i.e., not before bilateral lesions had been made. The most striking feature was a decrease in fear of humans. In some of the subjects this was expressed by docile behavior, including permitting and at times actual seeking of petting and scratching of all parts of the body, head and face. None however, became so tame as to tolerate the grasping of a limb, or attempts to lift and carry them. This always evoked vigorous biting, screaming and escape reactions. The exhibition of a toy snake, or the protective gloves used for capturing the animals, also induced normal fear reactions.

While some degree of docility was observed in all of the subjects, the decreased fear of humans in some of them was shown not so much by this, as by an increase in aggressively playful behavior. Previously timid animals, for example, became prone to strike at, and grasp, the clothing of human personnel passing their cages. If a hand were extended into the cage, such an animal would approach and explore it with his fingers, instead of showing the normal avoidance

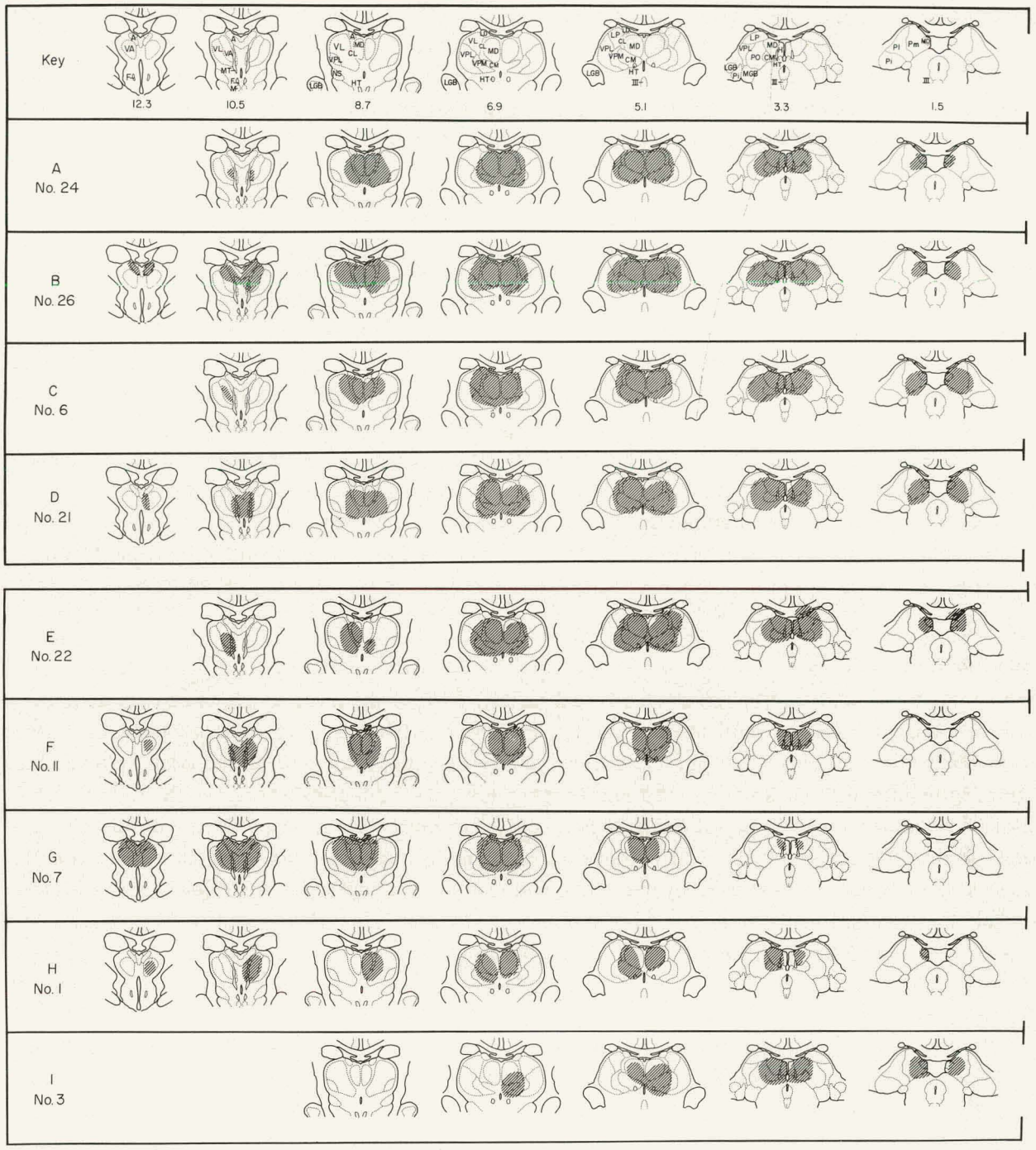

Figure 3. Diagrams of the thalamus in which the shaded portions represent areas of complete destruction. The dorsomedial nuclei in each animal are shown in their entire rostro-caudal extent. Levels free from lesions and rostral to the anterior extremities of the dorsomedial nuclei are omitted. The nuclear outlines are slightly modified from Olszewski.[14] The left and right sides in this and all succeeding illustrations correspond with those in the brain. In the key diagrams of the first row, the number below each is its frontal plane number, and the thalamic nuclei are designated by the conventional abbreviations. Other abbreviations are F, fornix; HT, habenulopenduncular tract; MT, mammillothalamic tract; NS, body of Luys; III, oculomotor nucleus.

reaction. A frequently observed response to a human hand held inside the cage was that of a gentle, rhythmically repetitive plucking at one of the fingers, which might persist automatically for some time even after the monkey's attention had wandered to something else. The animals were generally more placid than normal, their facial expressions exhibited a certain slackness, and the typical threatening attitude of the rhesus monkey, with fixed, frowning gaze and open mouth, was difficult to elicit.

All of the subjects showed these changes in general behavior. After the third month following surgery, they were no longer conspicuous, and the casual observer would then be unable to distinguish between a normal monkey and one with bilateral thalamic lesions. Two additional features of a more subtle nature, however, were noted in all of the subjects except monkeys no. 3 and 7, which persisted throughout the postoperative period of observation of 1 to 2 years. One of these was the gradual transformation of the placidity of the first few months to an emotional hyper-reactivity to frustrating situations. The other lasting behavioral alteration was a tendency to myoclonic startle reactions, and a certain excess and precipitancy of movement of the body and limbs, which perhaps correspond to the hyperactivity which has been described in monkeys following frontal cortical lesions.

Convulsive seizures were never observed. Casual observations disclosed no alterations in coital behavior in males or females. The cyclic edema and erythema of the skin of the tail and lower extremities associated with the menstrual cycle in the females appeared to continue normally.

Neurological Defects

Visual and ocular. Postoperative visual acuity in all of the animals appeared normal, as judged by their visual pursuit of, and orientation toward, tiny bits of food tossed to them. Visual fields were normal except for the transitory relative neglect of objects in the contralateral field already mentioned. Monkeys no. 3, 6 and 21 were the only subjects showing ocular disturbances. Nos. 6 and 21 had loss of pupillary light reflexes from involvement of the superior quadrigeminal brachia. Monkeys no. 3 and 21 had nystagmus, which was permanent in 21, but lasted only a few days in 3. In both cases the nystagmus developed after a lesion near the level of the middle of the dorsomedial nucleus, and in each instance there was ventral extension of the lesion into the pre-rubral field (Figures 3D and I). In addition to nystagmus and loss of pupillary light reflexes, monkey no. 21 also had paralysis of conjugate upward gaze, presumably from involvement of the rostral tectum (Figure 3D).

Motor and sensory defects in the extremities. Three of the subjects, monkeys no. 1, 3 and 11, had no abnormalities of posture or movement in the extremities. Of the others, 3 (nos. 6, 7 and 26) showed a unilateral, and 3 (nos. 21, 22 and 24) a bilateral disturbance in motor function of the upper extremities. This was evident mainly as a defect in manipulating small objects and in placing food in the mouth. This disorder of hand and finger movements was accompanied by a slight, almost imperceptible tremor of the affected hands when reaching for objects, and a curious disturbance in gait, manifested by exaggerated elevation of the affected upper extremity with each step. Strength in the arms and in the handclasps did not appear to be much diminished, and consistent alterations in muscle tone, or deep reflexes could not be detected. Only one animal (monkey no. 6) exhibited an abnormal posture of the hand.

Sensory loss was not found in any of the 3 monkeys with normal motor function in both hands.

In general, motor defects in the hands were accompanied by evidence of impaired sensation, particularly in the form of defective awareness, or recognition, of food and other objects placed in the hand outside the field of vision, and designated hereafter for convenience as impaired tactile sensation.

Impaired pain sensation was present bilaterally in the feet of all 4 monkeys who had hypalgesia in one or both hands (monkeys no. 6, 21, 26 and 24), and also in monkey no. 22, who had loss of tactile sensation only, confined to the right hand. Hopping and tactile placing reactions were rather delayed and slow in both lower extremities in all 5 monkeys with impaired pain sensation in the feet. In addition, one or the other of the hind limbs of these animals was often observed to assume an unnatural and awkward posture of extension at the knee and abduction at the hip in the seated position. On the other hand, visual placing reactions, weight-bearing, and movement of the lower extremities in walking, jumping and running appeared to be normal in all of the subjects.

None of the subjects was ever observed to exhibit behavior suggestive of spontaneous pain or hypersensitivity to sensory stimuli.

Facial, oral and taste sensation. Monkeys no. 24, 26, 21 and 22 showed weak responses to painful stimulation of the face. This correlated well with the extensive bilateral involvement of the posteromedial ventral nuclei which was found in these animals only (Figures 3A, B, D and E). Partially masticated food was often observed to drop from the mouths of 3 of them while feeding (monkeys no. 21, 22 and 24), indicating impairment of intra-oral as well as facial sensation. Loss of taste sensation was found only in monkeys 21 and 22, who were also the only animals in whom the medially situated parvocellular portions of the posteromedial ventral nuclei were completely destroyed on both sides (Figures 3D and E). This correlation is in keeping with other evidence pointing to the same region as a gustatory center in the monkey[20] and in man.[21] The gustatory impairment was profound in monkey no. 22, who avidly ate sections of banana soaked in saturated quinine solution. It was apparently incomplete in monkey no. 21, since this animal occasionally showed mild rejecting responses.

Deviation of the head and circling. An abnormal posture of the head was observed in monkeys no. 21, 22, 11 and 3. This consisted of a lateral tilt of the head toward one shoulder which appeared in marked form on the second or third postoperative day, and although persisting permanently, became quite mild after the first few weeks. The 4 subjects with dystonic postures of the head were the only ones in whom the lesions extended ventrally into the region of the prerubral field (Figures 3D, E, F and I). Similar deviations of the head were mentioned in the early descriptions of the effects of experimental diencephalic lesions, such as those by Bechterew,[22] Probst,[23] and Sachs.[24] A perusal of these reports indicates that the responsible lesions were also probably subthalamic.

"Forced" circling movements were also described by Bechterew and Probst. This was observed only in monkey no. 21 and ceased after a few weeks.

Delayed Response

All 3 of the animals with complete, or virtually complete, bilateral destruction of the dorsomedial nuclei (monkeys no. 6, 24 and 26) exhibited marked impairment in delayed response performance. Monkey no. 24, having achieved criterion at 30 secs delay preoperatively in 760 trials, failed to reach criterion at the minimum delay in 900 trials postoperatively (Figure 4A). The

last postoperative session in delayed response was given 5 months after the last pellet was implanted.

Monkey no. 6, who reached criterion at 20 seconds delay preoperatively in 760 trials, failed to reach criterion postoperatively at 5 secs delay in 940 trials (Figure 4C). Unlike monkey no. 24, this animal did reach criterion at minimum delay postoperatively, but required 500 trials to do so, in comparison to 240 trials preoperatively. Testing in delayed response was repeated in monkey no. 6 one year postoperatively. His performance did not improve after this length of time in spite of the use of the pre-delay exposure technique in the last 600 trials. The last session of the second attempt at retraining in delayed response was given 16 months postoperatively.

The third animal in the group with complete or nearly complete destruction of both dorsomedial nuclei, monkey no. 26, also showed marked impairment in delayed response, but not complete loss (Figure 4B). The last session of testing in delayed response was given 8 months after implantation of the last pellet.

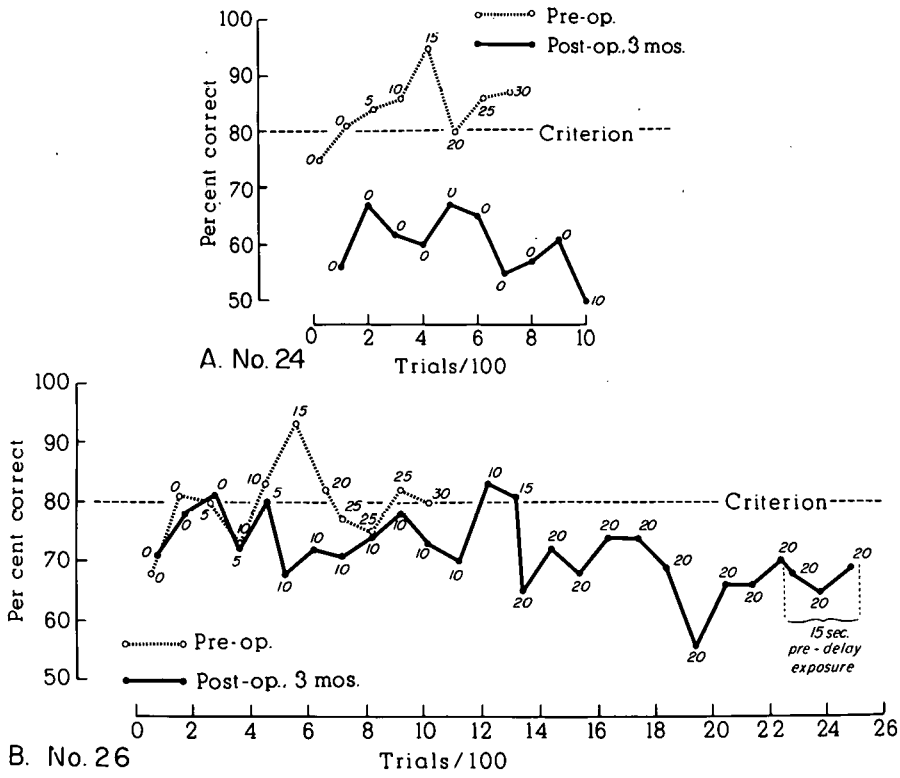

Figure 4. Pre- and postoperative learning curves in delayed response. Each point on the curves represents the score in per cent correct responses on a block of trials, and the number adjacent to each point indicates the delay interval at which the trials were run. Minimum delay trials are indicated by an adjacent cipher. An asterisk indicates that the trials were run under "open" conditions, i.e., the opaque screen was not lowered during the interval between baiting the positive cup and the response. In constructing the curves, all the trials run at a given delay were divided into blocks of 100, beginning with the criterion trials and proceeding backwards. The first point at a given delay, therefore, may represent a score based on a multiple of 20 trials less than 100, while all of the others represent scores on blocks of 100 trials. The postoperative time designations refer to the intervals elapsing between the implantation of the last pellet and the beginning of postoperative testing. The designation, "15 sec pre-delay exposure," indicates that on each trial the positive side was baited and the reward left exposed for 15 seconds before the cups were inverted over the choice points and the opaque screen lowered.

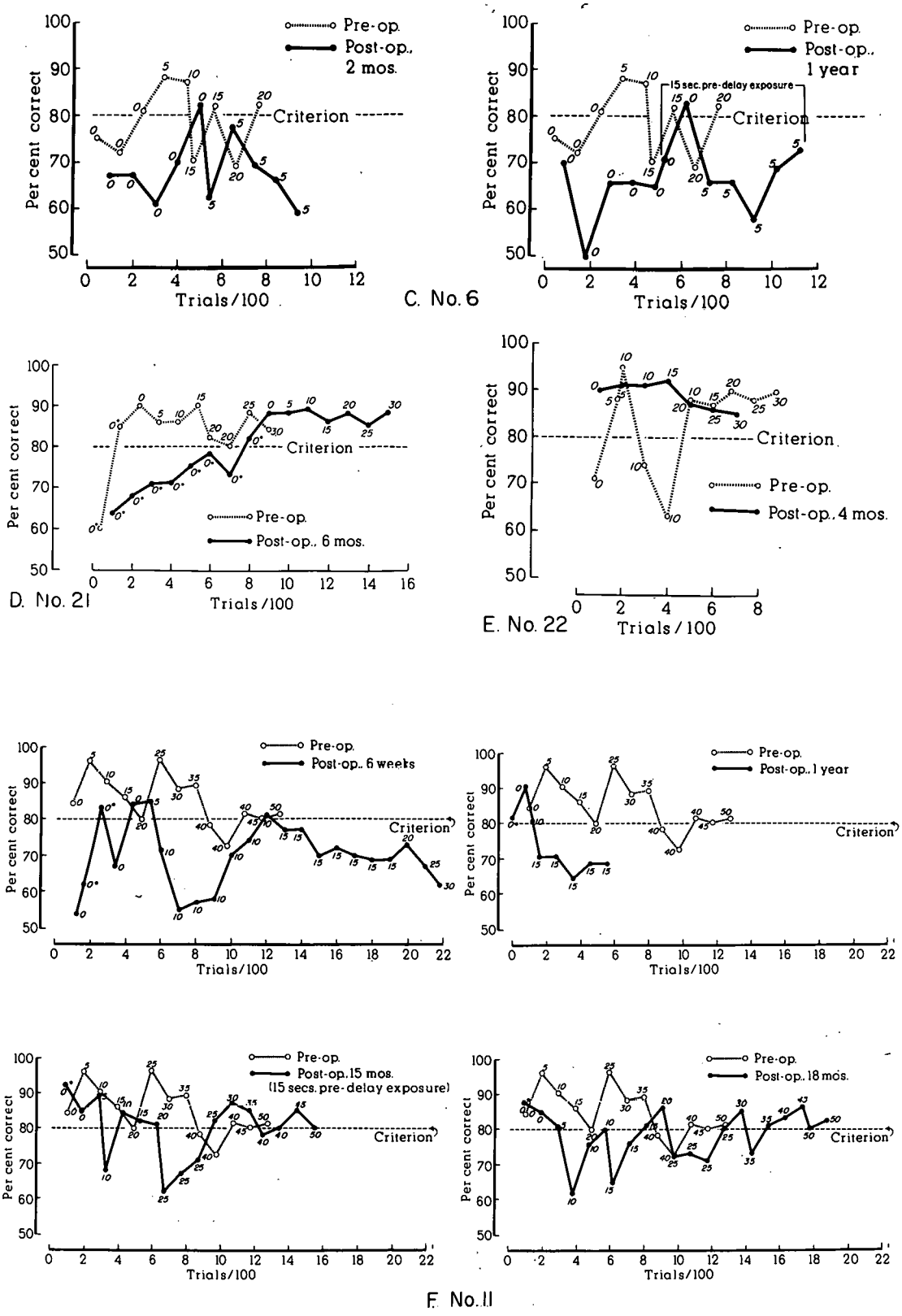

Figure 4. (continued)

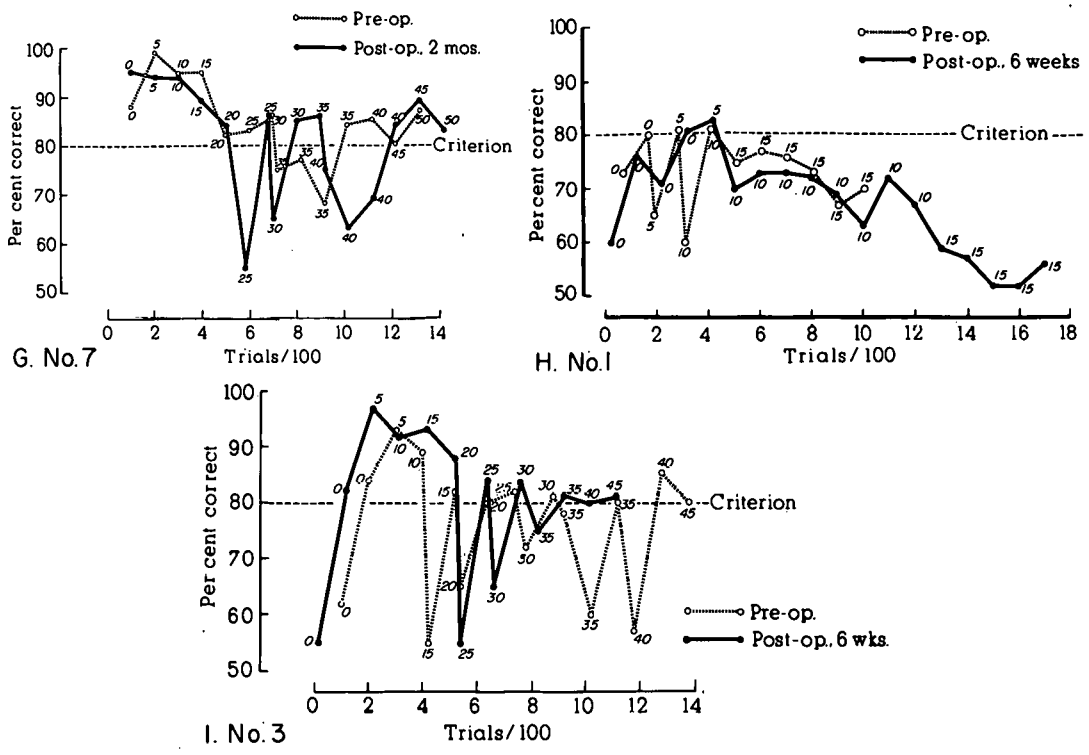

Figure 4. (continued)

Among the 6 animals with incomplete destruction of the dorsomedial nuclei, there was considerable variation in the postoperative delayed response results, which could not be consistently correlated with the degree of involvement of the nuclei (Figures 4D to I).

Visual Discrimination

The number of trials that were required by each monkey to reach criterion pre- and postoperatively for each of the 4 steps of the visual discrimination test, and the totals for all 4 steps are given in Table 2. Eight of the 9 subjects showed some degree of postoperative impairment in the visual discrimination test, but all of them eventually achieved criterion. Generally, the deficit appeared primarily in the first 2 steps, with insignificant losses or postoperative savings in the third and fourth steps.

There were no significant differences between the pre- and postoperative scores in the generalization tests, in which rectangles, diamonds, circles and crosses were used as the stimulus patterns. The mean of the grouped postoperative scores for all 9 monkeys was exactly the same as that of the preoperative scores: 75 per cent correct in the 80 critical trials of the test.

COMMENT AND CONCLUSIONS

The single most outstanding finding in these experiments was the severe and enduring impairment in delayed response performance in all 3 monkeys in whom the dorsomedial nuclei were completely, or nearly completely, destroyed bilaterally (monkeys no. 6, 24 and 26). No other thalamic nucleus could be correlated with the delayed response results in this way. Although the lesions in these 3 animals extended into other thalamic nuclei, similar involvement

Table 2

VISUAL DISCRIMINATION PROBLEM TRIALS TO CRITERION*

	Step 1		Step 2		Step 3		Step 4		Total	
Monkey	Pre-Op	Post-Op	Pre-Op	Post-Op	Pre-Op	Post-Op	Pre-Op	Post-Op	Pre-Op	Post-Op
24	225	950	125	650	50	100	0	50	400	1750
26	125	400	200	325	150	0	0	25	475	750
6	300	540	210	240	80	0	0	0	590	780
21	175	1000	275	500	1425	225	25	25	1900	1750
22	175	400	175	300	575	225	200	0	1125	925
11	210	540	120	120	40	40	0	0	370	700
7	180	120	210	60	200	40	0	30	590	250
1	30†	150	0†	60	0†	0	0†	0	30†	210
3	60†	90	0†	30	0†	0	0†	0	60†	120

*Excluding criterion trials.

†Pre-op retention.

could be found in animals showing little or no impairment in delayed response. To take what is perhaps the most important example of such overlapping, nucleus centrum medianum and most of the rest of the intralaminar system were extensively involved bilaterally in monkeys 6, 24 and 26, but this was also true in monkey 22, who showed postoperative savings in delayed response, and in monkey 21, who, except for an initial difficulty in reaching criterion at the minimum delay, also showed postoperative savings.

The impairment in delayed response was not related to the sensory-motor defects in the extremities of monkeys 6, 24 and 26, for these were quite mild, and present to an equivalent degree in animals without impaired delayed response. Only one of the 3 (monkey 24) had intra-oral sensory loss, and none of them had loss of taste sensation, while monkeys 21 and 22 showed signs of both.

During the first few months after operation, the changes in "personality" and temperament resembled those described by Brierley and Beck.[12] Later, however, the placidity was replaced by a somewhat decreased tolerance to frustration. Although these general behavioral changes were inconspicuous and short-lived in monkeys 3 and 7, they were present in all of the others to about the same extent, regardless of the presence or absence of impairment in delayed response.

Among the 6 animals with incomplete lesions of the dorsomedial nuclei, 5 showed either savings or slight impairment in delayed response, and 1, monkey no. 11, showed rather marked impairment, followed by improvement after extremely lengthy postoperative retraining. The delayed response performance in this group was not consistently related to the extent of the subtotal lesions. The most striking discrepancy was found in monkey no. 21, with what appeared to be negligible sparing of the dorsomedial nucleus on one side and 90 per cent destruction on the other. Nevertheless, after an unusual number of trials to reach criterion under "open minimum-delay" conditions, she showed postoperative savings in reaching criterion at 30 secs delay. The initial difficulty with the minimum delay trials may have resulted from the disturbance in extra-ocular muscle function, which was present in this animal, but in none of the others.

The external behavior of the subjects with impairment in delayed response was not greatly different in the test situation after operation than before. There was no evidence of amnesia for the procedure, and they appeared to be just as well motivated and eager to work. All of the traits which distinguished one animal from another prior to operation, such as the type and degree of activity during the delay interval, and the manner of observing the baiting of the positive side, were still present postoperatively. Although individual modes of responding were also preserved, such as customary rapidity and assurance, or vacillation and vicarious trial-and-error, there was a greater frequency of responses which seemed to depend on the chance orientation of the animal at the time the screens were raised, and a greater tendency to develop position preferences. It is worth mentioning, in view of the reports of Lawicka and Konorski[25] and Orbach and Fischer,[26] that none of the entire group of 9 animals was ever observed to use somatic orientation as a cue, either pre- or postoperatively.

Since the goal of total destruction of the dorsomedial nuclei was accomplished in only 3 animals, the conclusions suggested by the foregoing observations are proposed here provisionally. They are that the dorsomedial nucleus in monkeys is an essential component of the mechanism by which novel and transient events are represented and held centrally for relatively brief periods of time. There appears to be little or no regional localization within the nucleus in respect

to this function. Instead, a high degree of equipotentiality is exhibited, and a remarkably small portion of the nucleus may be sufficient for normal performance. Although in some animals incomplete lesions may produce deficits in delayed response, these are usually quite mild. What determines this individual variation in susceptibility to sub-total lesions is not clear.

Something of a paradox arises from equating the dorsomedial nucleus in this way with the frontal cortex, in respect to delayed response deficits. The sub-total frontal lesions that induce such deficits with the greatest regularity are those which involve the region of the sulcus principalis, and which are associated with rather restricted zones of retrograde degeneration in the dorsomedial nucleus.[27,28] If the latter does, in fact, play an important role in mediating delayed response, why do not similarly limited thalamic lesions cause deficits with equal regularity? This need not be the case even a priori, however, for the destruction of a thalamic region having an essential projection, in the sense of Rose and Woolsey,[29] to a given region in the cortex, does not necessarily mean that the cortex in question is deprived of all its connections with the thalamus.

Turning to the visual discrimination problem, the impairment observed was not of the type that might be anticipated from lesions of the dorsomedial nucleus. Difficulty in establishing the reversal in step 2, and in responding normally to the shifting cues in the third and fourth steps, might well have been attributed to interference with factors of a less specifically visual nature. These animals, however, although showing considerable impairment in step 2, had as much, or more, difficulty with the initial unambiguous discrimination in step 1, and none at all with the third and fourth steps.

It is possible that involvement of the medial nucleus of the pulvinar, a dependency of the temporal cortex, contributed to the disturbance in visual discrimination in the animals in whom the lesions extended into this structure. In monkey no. 1, however, it was involved only slightly and unilaterally, and in monkey no. 11, not at all—yet both showed impairment. The only subject with postoperative savings in the visual discrimination problem was monkey no. 7. Here, too, the pulvinar was intact bilaterally, but the dorsomedial nuclei showed a greater degree of sparing in their posterior portions than in any of the other subjects. It would seem, therefore, that the dorsomedial lesions were implicated in the impairment in the visual discrimination problem as well as in delayed response. This finding receives support from the recent report by Warren and Akert[8] of impairment in a similar conditional visual discrimination problem in cats with bilateral lesions of the dorsomedial nucleus. In their animals, the deficits were more equally distributed among the various stages of the problem, which in the later steps was more difficult than the one used here.

The deficits in visual discrimination that have occasionally been reported following lesions of the frontal cortex[26,28,30] are commonly attributed to disturbances in some such general factor as set, or vigilance, and a similar explanation might be entertained for the performance of the animals in the present study. It is not an altogether satisfactory one, however. The order in which the discrimination and the delayed response tests were given, which might be expected to influence set, or attitude, was without effect. Furthermore, although the animals did make frequent responses without appearing to compare the stimuli, comparison behavior was by no means completely lacking, even early in the course of postoperative testing, when the level of performance was still quite low. It is difficulty, therefore, to exclude some deterioration in the discrim-

ination itself, which is, after all, a complex and not a simple process, and from which the factor of set is not easily separable.

Whatever its precise mechanism, the impairment in the visual discrimination problem differed from that in delayed response in several respects. It was seen in all but one of the animals, rather than primarily in those with total lesions of the dorsomedial nuclei. It was not as severe; relearning always occurred, and preoperative overtraining appeared to increase the resistance of the habit to the effects of the lesions.

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REDUCTION IN RATE OF HAIR GROWTH IN MICE AS AN INDICATOR OF EXPOSURE TO CHRONIC LOW DOSAGE IONIZING RADIATION*

By

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Current biological criteria for assessing damage caused by chronic exposure to ionizing radiation include: life-span shortening, mortality rate, cataract formation, hematologic changes, incidence of tumors, and changes in certain enzyme activities.[1-4] Many of these changes develop slowly over long periods and require prolonged study of large numbers of animals. The present study reports observations which indicate that in mice, the rate of regrowth of hair after plucking is a sensitive, rapid and simple, dose-dependent biological index to damage caused by chronic, low dosage ionizing radiation.

MATERIALS AND METHODS

CF No. 1 female mice 7-8 weeks old at the onset of irradiation, were used in all experiments. Every time animals were taken out of the irradiation facilities for observation, a suitable number of non-irradiated animals were removed from the next room as controls.

Details of the chronic fast neutron and gamma radiation facilities, and the dosimetry factors have been described previously.[5,6] In brief, the gamma exposures were given by means of a 10-curie cobalt-60 point source, and the fast neutron exposures were administered by using a 100-curie plutonium-beryllium line source. The animals in groups of 12 were exposed for 10 h each day (9 PM to 7 AM) in metal or plastic cages placed radially around the sources. Irradiation was initiated by elevating the source from its storage pit into the center of the cage-array, and its duration was controlled by timing mechanisms which also controlled the source-hoisting motors. The animals remained in the exposure facility when the sources were shielded, since radiation exposure was negligible under these conditions. Food (Rockland Laboratory Diet) and water were available at all times. Radiation-doses in the gamma facility were determined with Victoreen Ionization Chambers which were calibrated against a radium standard, and against a Bureau of Standards calibrated chamber. Chemical dosimeters have also been used to verify the radiation dosimetry. The fast neutron dosimetry is based primarily on the manufacturer's specifications, with additional measurements with chemical dosimeters, with foils, and with a Hurst Proportional Counter. The gamma contamination within the fast neutron facility was estimated by a neutron-insensitive chemical system, and proved to be negligible.

In all animals, a 2 x 1.5 cm^2 area on the mid-dorsal region was epilated by hand-plucking to induce anagen, i.e., the growing phase of the hair cycle. The animals were then observed daily until a uniform sheen of new hairs appeared, which ordinarily occurred after 8 to 10 days. A small area of uniform new hair growth was outlined with a colored marker, and 6 days later the

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hair was again plucked from within this area. Three hundred to 400 of the plucked hairs were then placed in a Petri dish and floated on a small amount of water. Under a dissecting microscope, the 4 hair types indicated in Figure 1[7] could easily be identified. Intact, awl hairs were

Figure 1. Dry's four hair types in mice.

picked up with ophthalmological forceps and arranged parallel to one another on a standard 3-1/4 x 4" lantern slide glass plate. A 4 x 1 cm^2 strip of translucent graph paper (Keuffel and Esser No. 359T-14LG) was also placed on the glass plate which was then covered with another plate of the same size. After taping the 2 plates tightly together, the resulting "slide" was projected onto a screen giving an approximately 19-fold image enlargement.

In the magnified images, hairs damaged slightly in plucking could easily be recognized. About 10 per cent of the mounted hairs were so damaged and excluded from measurement. For each animal, the lengths of 35 straight hairs with intact anagen bulbs were measured with a Swiss map plan measure (Charles Bruning Company, Inc., Mount Prospect, Illinois, Catalogue No. 2375). Actual hair lengths were calculated by correcting their measured magnified lengths according to the magnification factor of the projected unit scale.

The animals irradiated with the cobalt-60 source received daily doses of 31.3 r gamma radiation for varying periods. At the time of the first epilation, i.e., 24 h after termination of irradiation, different groups of animals had accumulated total doses of 783 r to 3881 r at the rate or 31.3 rads/day.

RESULTS AND DISCUSSION

Table 1 shows that significant impairment of hair growth after induction of anagen is first observed after a total dosage of 1064 r has been accumulated, and increases thereafter in dose-dependent fashion at higher total dosage levels.

The animals irradiated with the plutonium-beryllium source received varying doses (from 35 to 595 rads) of fast neutron radiation at the rate of 3.18 rad/day. Table 2 shows that in these animals, the rate of hair growth is clearly retarded at a total dose of 80 rads. From there on, growth rates are progressively impaired as total dose increases.

Table 1

HAIR GROWTH RETARDATION IN ANIMALS GIVEN 10-HOUR DAILY DOSES OF 31.3 r GAMMA RADIATION IN THE 10-CURIE Co^{60} IRRADIATION FACILITY

Group no.	No. of animals examined	Total dose of radiation received in r	Mean length of hair for group in mm	Percentage retardation
GN-C	48	Nil	7.113 ± 0.036	——
G-I	8	783 (in 25 days)	7,033 ± 0.077	——
G-II	7	1,064 (in 34 days)	6.531 ± 0.061	8.1
G-III	7	1,314 (in 42 days)	6,045 ± 0.026	14.9
G-IV	4*	2,254 (in 72 days)	5.713 ± 0.054	19.6
G-V	7*	3,881 (in 124 days)	5.230 ± 0.030	26.4

*Three of 7 animals irradiated in Group G-IV and 7 of 14 in G-V died before completion of the experiment.

Table 2

HAIR GROWTH RETARDATION IN ANIMALS GIVEN 10-HOUR DAILY DOSES OF 3.18 rad FAST NEUTRON RADIATION IN THE 100-CURIE PLUTONIUM-BERYLLIUM IRRADIATION FACILITY

Group no.	No. of animals examined	Total dose of radiation received in rad	Mean length of hair for group in mm	Percentage retardation
GN-C	48	Nil	7.113 ± 0.036	——
N-I	9	35 (in 11 days)	7.081 ± 0.051	——
N-II	8	80 (in 25 days)	6.650 ± 0.042	6.4
N-III	8	117 (in 37 days)	6.529 ± 0.061	8.2
N-IV	7	229 (in 72 days)	6.228 ± 0.081	12.4
N-V	7	394 (in 124 days)	5.926 ± 0.023	16.6
N-VI	6	595 (in 187 days)	5.574 ± 0.034	21.5

Variations in hair length in individual animals were always less than 1.5 per cent of the mean length, and variations of the mean hair lengths between individuals in any one group were always under 1 per cent.

In the lower dosage ranges, where the general condition of the animals remained good, the relative biological effectiveness of chronic fast neutron radiation in retarding mouse hair growth was about 9 times that of gamma radiation. At the higher dose levels this RBE dropped sharply to about 4. The generally impaired nutritional state of these animals, together with smaller increments of hair growth retardation at these higher radiation dosages, may account for this difference.

The quite substantial retardation of hair growth at a dose level as low as 80 rads of fast neutron radiation given in 25 days, is noteworthy in view of the absence of other gross signs of radiation damage. Microscopic examination of hairs at all dosage levels studied failed to show dysplastic changes.[8,9]

This study indicates that hair growth rates serve as early, sensitive and simple, dose-dependent indices to chronic low dosage radiation. Further studies of the duration and degree of reversibility of these effects might be of interest. In addition, manifestation of these effects can be recognized early, and large numbers of animals are not required for statistically valid data.

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DEFICIENT CHOLESTEROL ESTERIFYING ABILITY OF LESION-FREE SKIN SURFACES IN PSORIATIC INDIVIDUALS*

By

A. Gara, E. Estrada, S. Rothman and A. L. Lorincz

Many lines of evidence support the view that keratinization and cholesterol metabolism in the epidermis are intimately connected. As early as the 1890's Liebreich[1] believed that the orderly formation of an epidermal horny layer requires an orderly arrangement of lipids. In 1910 Unna and Golodetz[2] first claimed that free cholesterol undergoes esterification during keratinization of epidermal cells in men. Kooyman[3] carefully re-examined and confirmed this finding in 1932.

More recently Swanbeck, using x-ray diffraction techniques, found evidence that the 250 Å protein fibrils of normal human horny layer are surrounded by a lipid layer about 80 Å thick,[4] and showed that this lipid layer is apparently altered under such conditions as exist in psoriasis.[5] Still further, it is recent history that clinically striking disturbances of keratinization have occurred as a complicating side effect from the dangerous drug triparsnol (MER-29) which blocks the reduction of the 24-25 double bond of the cholesterol precursors, desmosterol as well as lanosterol.

In 1950, Rothman[6] reported that the ratio of esterified to free cholesterol in the skin surface lipids of non-involved skin in psoriatic individuals was pathologically low, and postulated that predisposition to psoriasis might be associated with a genetically determined deficiency in the cholesterol esterifying ability of the keratinizing epidermis. Wheatly[7] later confirmed Rothman's findings, and also noted a decrease in unsaturated fatty acids in skin surface lipids from normal-appearing skin areas of psoriatics. He attempted to interpret these findings by postulating that the sebaceous glands produce mainly esterified cholesterol, while the epidermis produces mainly free cholesterol, some of which is esterified during the process of keratinization. In psoriasis, he felt that there was a decreased output of sebum, and an increased output of epidermal lipids, with the net result that the proportion of esterified cholesterol in the surface lipids of both the involved and uninvolved skin was lowered.

Simple non-specific esterases on the skin surface have been amply demonstrated. Steigleder[8] in particular made a detailed study of surface simple esterases, and considered as their possible sources sebum, sweat, keratinizing epithelial cells, and the skin's microbial flora, viewing the skin as having a protective "esterase mantle."

The current studies were undertaken to demonstrate directly cholesterol esterifying activity on the skin surface, and to compare this activity in normal and non-involved psoriatic skin.

MATERIALS AND METHODS

As indicated in Table 1, 11 normal and 9 psoriatic Caucasian volunteers served as experimental subjects. Fifteen-hundredths to 0.17 mg of palmitic acid containing 1 μc of carboxyl

*This report is taken from a paper presented before the Society for Investigative Dermatology at San Francisco, June 22-25, 1964 and published in J. Invest. Dermat., 43:559, 1964.

Table 1

DATA ON EXPERIMENTAL VOLUNTEERS

Subject	Sex	Age	Psoriatic manifestations at time of study
1	Male	60	Lesions chiefly on elbows and knees affecting less than 10% of skin surface.
2	Male	16	Psoriasis on scalp and pitting of nails.
3	Male	72	Lesions chiefly on hands and arms covering less than 15% of skin surface; nails affected.
4	Male	44	Chiefly intertriginous, elbow and lower extremity lesions affecting less than 10% of skin surface; nails affected.
5	Male	31	Guttate psoriasis affecting less than 10% of skin surface.
6	Female	54	Chiefly intertriginous, sacral and elbow lesions affecting less than 10% of skin surface.
7	Male	60	Psoriasis only on palms and fingers.
8	Female	46	Lesions on scalp, hands and left leg affecting less than 10% of skin surface.
9	Male	30	Psoriasis of nails.

All eleven of the subsequent subjects were normal males with ages as follows: 27, 33, 24, 32, 33, 25, 55, 29, 30, 50, 28.

C^{14}-label* dissolved in 2 ml of petroleum ether was pipetted dropwise evenly onto each of 2 demarcated areas of lesion-free skin approximately 300 cm^2 in area on the back of each subject. After 2.5 hours, during which time the outlined areas were freely exposed to the air while the subjects rested, each area was repeatedly thoroughly wiped, with petroleum ether-soaked cotton balls, until no further radioactivity could be detected on the skin surface with a Geiger, Eberline end-window counter, Model E-500B.

The total lipids were recovered from the cotton balls by 7 successive extractions in a separatory funnel with 100 ml portions of petroleum ether. These 7 extracts were pooled and washed successively with two 100- and two 50-ml portions of 0.06 N sodium hydroxide in 50 per cent ethanol in order to remove the free fatty acids from the neutral fat fraction.[9] These 4 washings were combined and counterwashed with two 50-ml portions of petroleum ether, and the counterwashings were combined with the original petroleum ether pool of neutral fats. This total neutral fat fraction was then dried under a stream of nitrogen.

The free fatty acid containing aqueous-alcohol phase was neutralized with 6 N sulfuric acid, and washed with successive two 100-ml and four 50-ml portions of petroleum ether to recover the non-esterified palmitic acid. This fraction gave a negative Liebermann-Burchard reaction.

Chromatographic separation of the neutral fat fraction was carried out on a silicic acid column essentially as described by Mukherjee.[10]

The neutral fat fraction, with 10 mg of non-labeled cholesterol palmitate as carrier, was

*New England Nuclear Corporation.

dissolved in 30 ml of pentane* and poured onto a 1 x 12.5 cm Unicil column (activated silicic acid, 100-200 mesh).† The column was then eluted with 50 ml each of 1, 4, 10, and 25 per cent ethyl ether in pentane, and 100 per cent ethyl ether. Each of these 5 eluates was collected in 15, 10, 10, and 15 ml serial subfractions off the column. An aliquot of each subfraction was tested for cholesterol and its esters by the modified Schoenheimer-Sperry method described by Windhorst and Foster[11] which is based on the Liebermann-Burchard color reaction. Optical densities for the determination of the free cholesterol and the cholesterol palmitate were read at 625 mμ on a Beckman DU spectrophotometer. Table 2 summarizes the chromatographic scheme and indicates some pertinent characteristics of the various fractions collected.

Table 2

CHROMATOGRAPHIC SCHEME AND CHARACTERISTICS OF COLLECTED FRACTIONS

Fraction no.	Eluent	Volume	Characteristics
		ml	
1	1% ether in pentane	15 10 10 15	Negative Liebermann-Burchard test Contains all cholesterol palmitate
2	4% ether in pentane	15 10 10 15	Negative Liebermann-Burchard test Contains all glyceryl tripalmitate
3	10% ether in pentane	15 10 10 15	Negative Liebermann-Burchard test
4	25% ether in pentane	15 10 10 15	Negative Liebermann-Burchard test Contains all free cholesterol
5	ether	15 10 10 15	Negative Liebermann-Burchard test

The isolated cholesterol ester containing fractions were further directly identified by drying 1-ml aliquots of the chromatographic cuts under a stream of nitrogen and taking up the residue in 3 to 5 ml of petroleum ether. Saponification was then carried out by adding a few drops of 10 per cent potassium hydroxide in 95 per cent ethanol and refluxing for 2 hours. After cooling, the liberated cholesterol was separated from the saponified fatty acids by extraction with petroleum ether. The fatty acid containing aqueous fraction was negative on Liebermann-Burchard testing, whereas the cholesterol containing petroleum ether fraction was positive. Only the fatty acid fraction contained radioactivity.

*Chromatoquality Reagent, Matheson, Coleman & Bill Company.

†Clarkson Chemical Company, Williamsport, Penn.

Radioactivities in all chromatographic fractions were measured in a Packard Tri-Carb Liquid Scintillation Counter. A 1-ml aliquot was taken from each collected fraction, dried under a stream of nitrogen and taken up in 15 ml of scintillation fluid consisting of 4 g PPO (2,5-Diphenyloxazole) and 0.1 g POPOP (1-4 bis-2-(5-Phenyloxazolyl)-Benzene) per liter of toluene.

In 4 normal volunteers, as an additional control, the entire study was repeated following a regimen of two daily baths and local applications of an ointment consisting of 6 per cent ammoniated mercury, 2.4 per cent salicylic acid, and 6 per cent Doak's tar distillate in aquaphor for 3 days to areas comparable to those which may have been similarly treated in some of the psoriatics just before the study. Because the microbial flora of the skin surface might have been implicated in cholesterol esterification, the study was repeated in 1 normal volunteer after pre-treatment of the test area with a neomycin, bacitracin, polymyxin-containing ointment (Neosporin ointment) for 1 day. Still more controls included 2 patients with minor eczematous dermatitis of the hands and feet, and 1 patient with an eczematized patch of dermatitis about a stasic ulcer.

RESULTS

Tables 3 and 4 indicate the percentages of the total recovered radioactivity in the cholesterol ester and glycerol ester fractions of the neutral fats obtained from the skin test areas in each normal and psoriatic subject.

In the 11 normal adults, from 1 to 25 per cent of the total radioactivity recovered was in the cholesterol ester fraction, the median being 4.9 per cent. In the 9 psoriatics, only 0.2 to 2.8 per cent of the recovered radioactivity was in the cholesterol esters, the median being 0.55 per cent. In general, there is an approximately ten-fold difference in cholesterol esterifying ability between the two groups. Table 5 indicates similar data for the additional control subjects. Results in all cases show skin surface cholesterol esterifying ability clearly in the normal range.

As concerns glycerol esterifying ability of the skin surface, no clear differences are apparent between the normal, control, and psoriatic subjects although there is possibly an equivocal trend toward diminished activity in the psoriatics.

DISCUSSION

The deficient cholesterol esterifying ability of the skin surface in lesion-free areas of psoriatic subjects might be based either on deficiency of the enzyme system required for cholesterol esterification, or on the presence of some inhibitor of this system. In either case this deficiency is probably genetically determined and may well be fundamental in determining predisposition to psoriasis, especially in view of the relationships between keratinization and epidermal cholesterol metabolism. Additional studies are under way on the cholesterol esterifying ability of the skin surface in the kindred of psoriatics and should give further information on the genetic dependence of this finding. Several speculations might be worth consideration at this point. For example, epidermal cholesterol synthesis in psoriatics may to a greater extent follow the Bloch pathway involving free zymosterol and desmosterol as precursors of free cholesterol, rather than the so-called Kandutch-Russell, probably normally predominant epidermal pathway, involving esterified lathosterol and 7-dehydrocholesterol as immediate precursor of esterified cholesterol. Reinertson's and Wheatley's[12] finding of deficient 7-dehydrocholesterol in the epidermal lipids of non-involved skin in psoriatics is consistent with this view, as is Wheatley's[7] finding

Table 3

EXPERIMENTAL RESULTS IN NORMAL SUBJECTS

Subject	Total counts	Counts in cholesterol esters	Counts in glycerol esters	Total counts in cholesterol esters	Ave.	Total counts in glycerol esters	Ave.
				%		%	
10	1,245,122 1,187,581	52,362 34,078	5,477 2,650	4.2 2.9	3.5	0.4 0.2	0.3
11	928,600 840,600	29,220 24,040	5,370 7,650	3.2 2.9	3.0	0.6 0.9	0.7
12	1,116,225 825,500	35,360 6,810	25,680 23,360	3.2 0.8	2.0	2.3 2.8	2.5
13	951,000 900,800	139,785 111,345	8,800 4,650	14.7 12.6	13.6	0.93 0.5	0.7
14	928,800 838,600	90,510 43,845	11,645 6,580	9.8 5.2	7.5	1.3 0.8	1.0
15	1,173,120 838,370	138,875 94,370	9,430 7,105	11.8 11.3	11.5	0.8 0.9	0.8
16	849,300 779,900	39,900 40,050	9,340 5,215	4.7 5.1	4.9	1.1 0.7	0.9
17	1,223,300 959,360	247,100 238,335	10,940 9,685	20.0 24.8	22.4	0.9 1.0	0.9
18	844,200 966,000	122,655 161,355	13,170 8,370	14.5 16.7	15.6	1.6 0.9	1.2
19	750,500 987,200	21,395 22,530	4,980 5,355	2.9 2.3	2.6	0.7 0.6	0.6
20	559,000 575,900	28,050 25,350	13,615 5,330	5.0 4.4	4.7	2.4 0.9	1.6

of a deficiency of unsaturated fatty acids in the surface lipids from non-involved skin of psoriatics. Cholesterol and its precursors are esterified with unsaturated fatty acids, and if such esterification is deficient, a smaller amount of unsaturated fatty acids would be carried to the skin surface during keratinization. Furthermore, in order to obtain the normal 80 Å lipid coat around keratin protofibrils suggested by Swanbeck's observations,[4] esterified cholesterol would probably be necessary and hence abnormal keratinization might be expected in the absence of sufficient cholesterol esters during keratin protofibril formation. If this be the basic defect in psoriasis, an obvious approach to rational therapy would aim at correcting the deficiency in epidermal sterol esterification: Perhaps even directly applying esters of cholesterol and its Kandutch-Russell precursors might be helpful.

The accelerated epidermopoiesis and its many consequences which are so characteristic of psoriasis could be secondary to defective keratin formation and loss of some normal feed-back suppression of epidermal cell division and differentiation. The speeded epidermopoiesis might

Table 4

EXPERIMENTAL RESULTS IN PSORIATIC SUBJECTS

Subject	Total counts	Counts in cholesterol esters	Counts in glycerol esters	Total counts in cholesterol esters	Ave.	Total counts in glycerol esters	Ave.
				%		%	
1	527,068 938,160	5,685 12,345	2,530 8,345	1.1 1.3	1.2	0.5 0.9	0.7
2	1,102,550 1,041,250	2,730 4,400	18,700 13,735	0.2 0.4	0.3	1.7 1.3	1.5
3	849,544 306,248	1,360 690	3,240 1,270	0.2 0.2	0.2	0.4 0.4	0.4
4	947,097 931,263	4,320 4,565	8,675 14,075	0.5 0.5	0.5	0.9 1.5	1.2
5	861,000 801,720	5,000 5,825	610 480	0.6 0.7	0.6	0.08 0.06	0.07
6	552,640 777,120	4,260 2,310	1,050 510	0.8 0.3	0.5	0.2 0.1	0.1
7	1,370,000 1,823,000	16,350 25,080	1,250 1,250	1.2 1.4	1.3	0.1 0.1	0.1
8	679,400 808,600	2,480 4,155	375 435	0.4 0.5	0.4	0.06 0.06	0.06
9	808,945 1,023,300	18,780 27,780	14,170 15,070	2.3 2.7	2.5	1.8 1.5	1.6

Table 5

EXPERIMENTAL RESULTS IN ADDITIONAL CONTROL SUBJECTS

Subject	Total counts	Counts in cholesterol esters	Counts in glycerol esters	Total counts in cholesterol esters	Ave.	Total counts in glycerol esters	Ave.
Normal subjects after antipsoriatic treatment regimen							
				%		%	
11	1,698,000 1,898,400	39,225 55,710	9,090 8,200	2.3 2.9	2.6	0.54 0.44	0.5
10	1,011,840 857,560	123,940 63,700	7,410 4,290	12.3 7.4	9.8	0.7 0.5	0.6
16	1,365,400 1,315,000	66,016 74,840	5,520 3,730	4.8 5.7	5.2	0.4 0.3	0.3
18	1,018,050 1,169,520	46,310 38,560	5,180 3,465	4.6 3.3	3.9	0.5 0.3	0.4
neosporin 10	1,476,000	42,368	15,321	2.9		1.0	
Subjects with minor eczematous dermatitis							
21	930,600 860,090	15,155 27,780	395 695	1.7 3.2	2.4	0.1 0.1	0.1
22	657,500 731,625	41,740 83,220	7,170 10,980	6.3 11.4	8.8	1.1 0.8	0.9
23	881,430 1,058,860	72,350 33,440	36,572 9,582	8.2 3.2	5.7	4.2 0.9	2.5

even be related to allergic so-called autosensitization to abnormal antigens arising during the defective keratinization process.

Under any circumstances the epidermis in psoriatics, because of its deficient cholesterol esterifying ability, might not be able to form sufficient normal aggregated keratin under the increased demand for keratin production that occurs wherever epidermopoiesis is accelerated from any cause. The predilectional localization of psoriasis to areas of physiologically most active epidermopoiesis[13] as well as the Koebner phenomenon could be accounted for in this way. Conversely, the beneficial effect on psoriatic lesions of any agent or procedure which inhibits epidermopoiesis could also easily be understood.

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HOMEOSTASIS OF ANTIBODY FORMATION IN THE ADULT RAT*

By

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Passive immunization of rats with homologous anti-sheep erythrocyte serum markedly inhibits the primary antibody response to sheep erythrocytes, and the unresponsiveness induced by a single passive immunization is sustained by weekly injections of this antigen.[1]

The mechanism producing sustained immune unresponsiveness in adult animals by this simple procedure has not been elucidated but experiments to be reported in this paper suggest that specific antibody adsorbed onto or incorporated into potential antibody forming cells inhibits these cells from forming specific antibody. Antibody does not have this effect on cells from actively immunized rats. Thus, the formation of specific antibody may provide a homeostatic or "feed-back" mechanism which controls or limits production of specific antibody to that portion of the antibody-forming system previously stimulated by the antigen. This mechanism may account in part for immunological unresponsiveness produced in mature animals by various procedures.

MATERIALS AND METHODS

Young adult male Sprague-Dawley rats, weighing 180 to 250 gm were fed Purina Chow and water ad libitum. All injections were in the lateral tail vein, and unless otherwise stated, 1.0 ml in volume.

Antigens and antisera. The antigens were washed whole sheep erythrocytes[1] or flagella of Salmonella typhosa.[2] Erythrocyte suspensions were prepared from packed cells, and their concentrations were standardized by hemoglobin determinations. Repeated cell counts showed that a 1 per cent suspension of sheep erythrocytes contained about 2×10^8 cells/ml. Cell counts were always made when dosages are reported as numbers of erythrocytes.

Pooled rat anti-sheep erythrocyte serum was obtained from a large group of rats exsanguinated 6 days after receiving a single injection of 0.25 per cent sheep erythrocytes. Pooled rat anti-flagellar serum was obtained from rats exsanguinated 6 days after an injection containing 10 μg protein of the flagellar antigen. The pooled sera were stored at -30° C until used. Antibody titrations against sheep erythrocytes or whole killed S. typhosa were carried out as previously described using double dilution technique;[1,2] the initial serum dilution being 1:10. The titers were recorded as the number of the last tube showing complete hemolysis of sheep erythrocytes or the number of the last tube containing grossly visible aggregations of S. typhosa; thus, the titers expressed the serum dilution as 10×2^n where n is the tube number. On repeat-

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ed titrations the pooled anti-sheep erythrocyte serum had a titer of 7 to 8, and the pooled anti-flagellar serum had a titer of 6 to 7.

In some experiments sera were treated with 2-mercaptoethanol (2-ME) for antibody titrations or for passive immunizations. For these titrations, sera diluted 1:5 in saline were added to an equal volume of 0.1 M 2 ME and incubated at 37° C for 30 minutes:[3] The initial serum dilution was 1:10 and the subsequent titration was the same as for untreated sera. For passive immunizations, 10 ml of antiserum diluted 1:5 in saline was added to an equal volume of 0.1 M 2 ME and incubated at 37° C for 30 minutes; the mixture was restored to original volume using pressure dialysis in the cold. The antibody inactivated by 2 ME will be referred to as "19S" antibody, and the antibody not inactivated by 2 ME as "7S" antibody.

At low serum dilutions the serum 2 ME mixture was anticomplementary; at higher serum-2 ME dilutions 2 ME did not affect titers. In order to titrate for low concentrations of "7S" antibody, hemagglutinin titers were done. Serum dilutions were as for hemolysin titers but were made in saline containing 1 per cent inactivated normal rat serum. Two tenths ml of 0.25 per cent suspension of sheep erythrocytes was added to each 13 x 100 mm tube containing 0.3 ml of serum dilution. The tubes were shaken briefly and incubated at room temperature overnight. The agglutination pattern equivalent to complete hemolysis was characterized by an evenly dispersed film of erythrocytes covering the bottom of the tubes, sometimes with crinkling of the margins of the film; the highest serum dilution showing this pattern was considered the end point. Many sera containing predominantly "19S" or a mixture of "19S" and "7S" antibodies were titrated by both the hemolysin and agglutinin techniques. Sera untreated with 2 ME and titrated by the two methods had the same titers plus or minus one tube number. The presence of 2 ME in low serum dilutions did not measurably affect hemagglutination. Hemolysin titers are reported in Tables 1, 2, 4 and 5, hemagglutinin titers in Tables 3 and 6. Mean titers for groups of rats ± the standard error of the mean are reported in Tables 1 to 5; a titer of 0 was arbitrarily assigned to sera showing less than the end point reaction to the antigen at a serum dilution of 1:10. Titers for sera treated with 2 ME are reported only when the results are directly pertinent.

Spleen cells and X-irradiated recipients. The capacity of spleen cells to form antibody was measured by transferring the cells to rats made unresponsive by X-irradiation. The donor rats were decapitated. Each spleen was minced and passed through a #80 tantalum gauze screen using a pestle and 4.0 ml of a modified tissue culture medium.[4] The same medium was used for washing and diluting the spleen cells. Tissue fragments and large aggregates of cells were removed by low speed centrifugation for 30 seconds. The spleen cells were washed twice. The number of nucleated cells was determined by hemocytometer counts and the suspensions were diluted to contain the desired number of nucleated cells per ml of suspension. In most experiments a yield of 2 to 3 x 10^8 nucleated spleen cells was obtained from each donor spleen. Spleen cells were prepared at room temperature. No immediate or late untoward effect was ever observed in any rat receiving the spleen cell suspensions. Irradiated rats received 600 r total body X-irradiation given as a single dose by a 250-KVP machine operating at 15 ma with 0.25 mm Cu and 1 mm Al added filtration. The dose rate averaged 39 rads per minute at 75 cm.

Plaque forming cells and plate hemolysin titers. The method of Jerne and Nordin for demonstrating release of antibody by single cells in agar plates was used with minor modifications.[5] Tissue culture medium[4] containing no serum was used for preparing spleen cell and sheep eryth-

rocyte suspensions and agar plates. DEAE-dextran was added to the agar solutions.[6] Two ml of fresh normal rat serum diluted 1:3 in normal saline was used for complement. Plates were incubated for 1 hour at 37° C before addition of complement, and for 1 hour afterwards. Plates were rinsed in normal saline after incubation and were counted immediately using a dissecting microscope, magnification 10 to 25 X, and dull transillumination of the plates. Agar solutions, cell suspensions, and sheep erythrocytes were as originally described.[5]

Each rat spleen was minced and passed through a #80 tantalum gauze screen using a pestle and 4.0 ml of tissue culture medium. The volume was brought to 5.0 ml and tissue fragments and large aggregates of cells were removed by low speed centrifugation for 30 seconds. The number of nucleated cells in the suspension was determined by hemocytometer counts. Plates were prepared in triplicate from the undiluted suspension, and from 3 five-fold serial dilutions. The total number of plaque forming cells per spleen or per 10^6 recovered spleen cells was calculated from the average plaque count of at least 3 plates.

The agar plate technique was sufficiently sensitive to demonstrate hemolytic antibody released by single cells. Quite reasonably, agar plates containing a thin layer of sheep erythrocytes could be used as a sensitive method for demonstrating anti-sheep erythrocyte antibody in serum. Agar plates were prepared as above but only sheep erythrocytes were added to the thin overlayer. Microdroplets of about 0.005 ml of undiluted inactivated serum and of serial 2-fold dilutions of the serum were spotted on the plates which were incubated at 37° C for 30 minutes, then rinsed several times with saline. Two ml of fresh rat serum diluted 1:3 in saline were added as complement, and the plates were incubated again at 37° C for 30 minutes. Clear "plaques" of complete hemolysis about 2 to 3 mm in diameter which were considered a 4+ reaction were produced by serum dilutions 2 to 4 fold higher than those which produced 4+ reactions using the tube hemolysin or hemagglutinin techniques. Higher serum dilutions produced distinct plaques of partial hemolysis which could be arbitrarily ranked as 3+ to 1+ reactions. Since only small volumes of serum were required, it was possible to begin titrations with undiluted serum.

Histological preparations. 1 mm-thick transverse slices of spleen were fixed for 2 hours in Carnoy's alcohol-acetic acid-chloroform mixture, embedded in paraffin, sectioned at 5 μ, and stained with methyl green-pyronine.[7]

PRELIMINARY EXPERIMENTS

The disappearance of passively transferred antibody. In four different experiments a total of 28 rats were injected with 1.0 ml of the anti-sheep erythrocyte serum, and were bled 1, 4 and 6 days later. At 1 day the mean titer for all rats was 2.3, at 4 days the mean titer was 0.3, at 6 days the mean titer was 0. The half life of circulating passively transferred antibody was about 1.5 days. Thus, in subsequent experiments, antibody titers of sera obtained 5 or more days after both passive and active immunization against sheep erythrocytes measured actively formed antibody.

Inhibition of the antibody response to various doses of erythrocytes. Twelve rats were passively immunized with 1.0 ml injections of anti-sheep erythrocyte serum; 24 hours later 4 rats were given injections of 0.25 per cent, 4 rats of 2 per cent, and 4 rats of 4 per cent sheep erythrocytes. Twelve nonpassively immunized rats (controls) were similarly injected. The mean 6 day titers for the passively immunized rats were 0.4, 1.0 and 1.5 and for the control rats 8,

7.5 and 8.2. Titers for all rats were lower at 10 to 14 days. Thus, the determination of titers at 6 days, the time of peak antibody response, accurately demonstrated inhibition of antibody formation produced by passive immunization.

Specificity of inhibition of antibody formation. Rats, passively immunized with either anti-sheep erythrocyte serum or with anti S. typhosa serum, were actively immunized 24 hours later with sheep erythrocytes or with flagellar antigen. Table 1 demonstrates that the anti-sheep erythrocyte serum inhibited the antibody response to sheep erythrocytes but not to S. typhosa. Similarly, the anti-flagellar serum inhibited the agglutinin response to S. typhosa but not to sheep erythrocytes. Passively transferred antibody to S. typhosa disappeared slowly and was present in rats which received the antisera only and were bled 6 and 14 days later.

Table 1

THE SPECIFICITY OF INHIBITION OF ANTIBODY RESPONSE BY PASSIVE IMMUNIZATION

Passive immunization with:		Anti-sheep RBC	Anti-sheep RBC	Anti-flagella	Anti-flagella	Anti-flagella	Anti-sheep RBC
Active immunization with:		None	Sheep RBC	Sheep RBC	None	Flagella	Flagella
Antibody titers to sheep RBC*	6 day	0	0.4 ± 0.24	4.6 ± 0.40	-	-	-
	14 day	0	0	2.4 ± 0.51	-	-	-
Antibody titers to S. typhosa*	6 day	-	-	-	4 ± 0.32	3.8 ± 0.20	6.5 ± 0.29
	14 day	-	-	-	2.5 ± 0.29	2.5 ± 0.64	5.5 ± 1.2

*Mean titers for groups of 5 rats ± the standard error of the mean.

Inhibition of the antibody response by "19S" or "7S" antibody. The pooled anti-sheep erythrocyte serum had a titer of 7 to 8. After treatment with 2 ME the anti-serum had no measurable titer at a dilution of 1:10, indicating that the serum contained predominantly "19S" antibody. Rats were passively immunized with either the antiserum or the antiserum treated with 2 ME. They were actively immunized 24 hours later with 0.25 per cent sheep erythrocytes and were bled at 6 days. The untreated serum markedly suppressed the antibody response, while the 2 ME treated serum had no effect. Thus passive immunization with "19S" antibody suppressed the primary response, but gave no information on the effect of passive immunization with "7S" antibody.

A second pool of rat anti-sheep erythrocyte serum was obtained from rats given injections of 0.25 per cent sheep erythrocytes every 3 or 4 days for a total of 8 injections and exsanguinated 4 days after the last injection. The titer of the pooled serum was 8, and after treatment with 2 ME the titer was 6, indicating that the serum contained a mixture of "19S" and "7S" hemolysin. Five rats were passively immunized with this serum containing a mixture of "19S" and "7S" antibody, 5 rats with the same serum treated with 2 ME and containing predominantly "7S" antibody; and 5 rats with the primary response anti-serum containing predominantly "19S" antibody. These rats, plus 5 additional non passively immunized rats were actively immunized with 0.25 per cent sheep erythrocytes 24 hours after passive immunization, and at 6 day inter-

vals thereafter for a total of 4 injections. They were bled for titers 6 days after each active immunization. i.e., just before the second, third and fourth active immunizations.

The 3 antisera were equally effective in markedly suppressing the primary hemolysin response. Furthermore, markedly sustained suppression occurred in all rats in each passively immunized group. In contrast, the non-passively immunized rats had high titers at all bleedings. Thus "19S", "7S" or a mixture of "19S" and "7S" antibodies, were equally effective in suppressing the primary response, and in producing sustained suppression when injections of antigen were continued. The experiments did not exclude the possibility that suppression may have been due to a different antibody fraction. Only the pooled primary response anti-sheep erythrocyte serum, containing predominantly "19S" antibody, was used in subsequent experiments.

Inhibition of the splenic cellular response by passive immunization. The spleen is essential for hemolysin formation after intravenous injection of "low" doses of sheep erythrocytes into rats.[8] Sheep erythrocytes and various other particulate antigens given via this route cause proliferation of pyroninophilic cells in spleens.[7] X-irradiation and certain cytotoxic agents, which depress antibody formation, inhibit this cellular proliferation.[7] It was of interest to determine whether the apparently innocuous passive immunization with homologous antiserum would similarly inhibit splenic cellular proliferation associated with antigenic stimulation.

Rats were passively immunized with anti-sheep erythrocyte serum and 24 hours later were injected with 0.5 per cent, 2 per cent or 4 per cent sheep erythrocytes. Control rats received antiserum only, sheep erythrocytes only, or no injections. Rats were bled and sacrificed 4 to 8 days after active immunization. Spleens were weighed and sampled for histological studies.

Spleen weights of rats injected with 2 per cent or 4 per cent sheep erythrocytes and sacrificed 4 or 6 days later averaged 100 to 200 mg heavier than spleens of passively immunized rats which received similar doses of antigen, antiserum alone, or no injections. The antigen alone caused the typical response of increased numbers of pyroninophilic cells at the margins of lymphoid follicles and enlarged clusters of pyroninophilic cells in the red pulp. The spleens of rats that were passively immunized before active immunization, or simply passively immunized, were histologically the same as those of noninjected control rats. Thus, passive immunization before active immunization inhibited the increase in spleen size and the hyperplasia of pyroninophilic cells in the spleen as well as inhibiting the antibody response. See Table 2.

Inhibition of the antibody response by passive immunization after active immunization. The previous observation that passive immunization after active immunization inhibited hemolysin formation[1] was confirmed. Groups of 5 rats were passively immunized with anti-sheep erythrocyte serum 24 hours before or after active immunization with 0.25 per cent sheep erythrocytes. A third group of 5 rats were actively immunized without passive immunization. The mean 6 day titers were 0.4 for rats passively immunized before active immunization, and 0.8 for rats passively immunized after active immunization. The mean titer for the non passively immunized rats was 5.

EXPERIMENTAL OBSERVATIONS

Certain parameters of the phenomenon of suppression of the primary immune response by passive immunization were explored in the preliminary experiments. Passive immunization suppressed the cellular proliferation associated with antibody production, and this suggested that passive immunization inhibited antibody formation rather than its release. Passively given anti-

Table 2

THE EFFECT OF ACTIVE IMMUNIZATION ON THE SPLENIC HYPERPLASIA AND ANTIBODY RESPONSE OF NORMAL AND PASSIVELY IMMUNIZED RATS

	Spleen weights in grams*	Pyroninophilic hyperplasia*	Mean antibody titers*
Active immunization†	0.97 ± 0.06	3 to 4 +	6.6 ± 0.61
Passive immunization before active immunization‡	0.79 ± 0.03	1 to 2 +	1.3 ± 0.36
No immunization	0.81 ± 0.03	1 to 2 +	0 ± 0

*Mean spleen weights and titers for groups of 7 rats ± the standard error of the mean. Hyperplasia of pyroninophilic cells was estimated from observations on multiple sections of spleen from each rat.

†Rats immunized with 1.0 ml of 2.0% suspension of sheep erythrocytes and sacrificed 6 days later.

‡Passive immunization with 1.0 ml of anti-sheep erythrocyte serum 24 hours before active immunization.

body and antigen may have formed complexes which altered the antigenicity of the antigen or altered its distribution. However, small i.v. doses of particulate antigen are cleared from the circulation rapidly, and passive immunization as late as 24 hours after active immunization was effective in suppressing the immune response. Therefore, the formation of complexes between passively given antibody and intact circulating antigen was presumably not necessary for suppression of the antibody response.

A secondary hemolysin response was demonstrated in rats 3 to 4 weeks after primary immunization, when the titers of circulating hemolysin were low. As might be expected, passive immunization of previously immunized rats did not suppress the secondary hemolysin response.[1] Apparently, the previously immunized animal had an altered capacity to respond to antigen in the presence of circulating antibody.

Experiments with passive immunization of intact animals gave no information concerning the mechanism of suppression of the primary response. Experiments were undertaken to determine whether circulating or cell bound antibody produced the inhibition of antibody formation. The differential effect of passive immunization on the primary and secondary responses was explored.

Primary and secondary antibody response to sheep erythrocyte-antibody complexes. Sheep erythrocyte-antibody complexes were produced in vitro. In one experiment 4.0 ml of antisheep erythrocyte serum inactivated at 56° C for 30 minutes was added to 2.0 ml of 5 per cent sheep erythrocytes. (Four ml of the anti-serum was estimated sufficient for complete hemolysis of about 400 times the number of sheep erythrocytes in 2.0 ml of 5 per cent sheep erythrocytes in the presence of sufficient complement.) The suspension was incubated at 37° C for 15 minutes; hemolysis was absent but agglutination of erythrocytes occurred. The cells were washed 3 times in large volumes of normal saline, and mechanically dispersed after each washing by repeatedly

passing the suspension through a fine needle. After the last washing the cells were resuspended in a volume of 20 ml of saline to give the equivalent of a 0.5 per cent suspension of sheep erythrocytes. The suspension contained predominantly singly dispersed cells and only rare small aggregates of erythrocytes. The supernatant fluid of an aliquot of the washed suspension contained no measurable antibody. Although complexes were produced in the antibody excess, free antibody was removed. A second suspension of erythrocytes was prepared in an identical manner except that the cells were incubated with inactivated normal rat serum.

The antigenicity of the 2 suspensions was compared in normal rats and in rats immunized 4 weeks previously with 0.25 per cent sheep erythrocytes. The previously immunized rats had titers of 2 or less at the time of re-immunization. Five normal and 5 previously immunized rats were given injections of 0.5 per cent suspension of erythrocytes incubated with immune serum and 5 normal and 5 previously immunized rats were injected with 0.5 per cent suspension of erythrocytes incubated with normal serum. The 6 day titers, see Table 3, show that the erythrocyte-antibody complexes failed to elicit a measurable response in normal rats but produced a high secondary response in the previously immunized rats.

Table 3
PRIMARY AND SECONDARY ANTIBODY-RESPONSE TO SHEEP ERYTHROCYTE-ANTIBODY COMPLEXES

	Immunization†	6 day Antibody Titers*
Primary response‡	Sheep erythrocytes	6.2 ± 0.80
Primary response‡	Sheep erythrocyte-antibody complexes	0 ± 0
Secondary response§	Sheep erythrocytes	7 ± 0.55
Secondary response§	Sheep erythrocyte-antibody complexes	5 ± 0.55

*Mean titers for groups of 5 rats ± the standard error of the mean.

†1.0 ml of 0.5% suspension of sheep erythrocytes injected intravenously. The erythrocytes, after incubation with normal rat serum or with anti-sheep erythrocyte serum, were washed and resuspended in saline.

‡Normal rats.

§Rats immunized 4 weeks previously with 1.0 ml of 0.25% sheep erythrocytes.

Another experiment was of similar design except that erythrocytes were incubated with antiserum which was not inactivated. The erythrocytes were completely hemolyzed, the stromata were washed and resuspended as in the previous experiment. The erythrocyte stroma-antibody complexes failed to elicit an antibody response in normal rats but produced a high secondary response in previously immunized rats.

Thus the suppression of antibody formation produced by passive immunization prior to active immunization might result from formation of antigen-antibody complexes in the circulation. Apparently such complexes formed in the circulation would not stimulate a primary response but would elicit a secondary response. The following experiments were designed to determine if antibody bound to spleen cells of non-immunized rats might also be effective in suppressing the primary antibody response of these cells.

The effect of passive immunization on the primary antibody response of normal spleen cells transferred to X-irradiated rats. A series of experiments was designed to measure the effect of passive immunization on the primary response of normal spleen cells transferred to rats made immunologically unresponsive by X-irradiation. Spleen cells were "passively immunized": 1) after injection into recipient rats, 2) in vitro or 3) in donor rats. The X-irradiated recipient rats were injected with spleen cells 3 to 4 hours after X-irradiation and were actively immunized 24 hours later. The rats were bled for antibody titers 6 days after active immunization. Each experiment included 3 control groups of 5 rats: X-irradiated rats not injected with spleen cells but actively immunized with sheep erythrocytes; non-irradiated rats passively immunized with anti-sheep erythrocyte serum before active immunization; and non-irradiated rats actively immunized with sheep erythrocytes. The 6 day antibody titers for the control groups invariably showed: no measurable antibody titer for the X-irradiated rats; no, or very low titers for the passively immunized non-irradiated rats; and high titers for the non-irradiated rats. Other control groups were usually included.

The following experiment demonstrated that suppression of the hemolysin response could be reproduced with the spleen cell transfer system. Ten X-irradiated rats were injected with 5×10^8 nucleated spleen cells from normal rats. Next day, 5 of the rats were injected with anti-sheep erythrocyte serum, and two hours later all rats were actively immunized with 0.5 per cent suspension of sheep erythrocytes. The results, (Exp. 1 Table 4) showed that X-irradiated rats receiving normal spleen cells had moderate 6 day titers, and that passive immunization inhibited this response. Since this experiment did not indicate whether circulating or cell-bound antibody was responsible for the inhibition, the following experiments were designed.

Table 4

THE EFFECT OF PASSIVE IMMUNIZATION ON THE PRIMARY ANTIBODY RESPONSE OF NORMAL SPLEEN CELLS TRANSFERRED TO X-IRRADIATED RATS

		Spleen cells transferred to X-irradiated rats†	6 day antibody titer*
Exp. 1	Passive immunization of spleen cells in recipient rats	Normal cells‡	3.2 ± 0.58
		Passively immunized cells‡	0 ± 0
Exp. 2	Passive immunization of spleen cells in vitro	Normal cells§	3.4 ± 0.68
		Passively immunized cells§	0.6 ± 0.60
Exp. 3	Passive immunization of spleen cells in donor rats	Normal cells¶	3.6 ± 0.93
		Passively immunized cells¶	1.0 ± 0.63

*Mean titers for groups of 5 rats ± the standard error of the mean.

†Recipient rats received 600 R total body irradiation and were actively immunized with 1.0 ml of 0.5% suspension of sheep erythrocytes 24 hours after injection of spleen cells.

‡5×10^8 nucleated spleen cells transferred to each X-irradiated rat.

§1.6×10^8 nucleated spleen cells transferred to each X-irradiated rat.

¶1.0×10^8 nucleated spleen cells transferred to each X-irradiated rat.

Washed spleen cells pooled from normal donor rats were divided into two equal 5.0 ml samples; 5.0 ml of rat anti-sheep erythrocyte serum was added to one sample, and 5.0 ml of normal rat serum to the other. After incubation with the antiserum for 15 minutes at room temperature each cell suspension was centrifuged and washed twice in large volumes of medium. After the second washing the volume of each suspension was adjusted to contain 1.6×10^8 cells per ml. Five X-irradiated and 5 non-irradiated rats were injected with the cell suspension which had been incubated with the anti-sheep erythrocyte serum, while 5 X-irradiated and 5 non-irradiated rats were injected with the other cell suspension. The following day all rats were actively immunized with a 0.5 per cent suspension of sheep erythrocytes.

The results, (Exp. 2 Table 4) showed that recipients receiving spleen cells incubated with the normal serum all responded with moderate antibody titers of from 1 to 4. In contrast, the recipients of spleen cells incubated with anti-sheep erythrocyte serum had no or very low titers. Control groups included non-irradiated rats which received the spleen cell suspension and were actively immunized; these rats had hemolysin titers equal to the titers of activcly immunized non-irradiated controls, showing that the spleen cells "passively immunized" in vitro and then washed did not carry sufficient antibody to affect the primary antibody response of normal rats. Moreover, the supernatant fluid of an aliquot of the washed, "passively immunized" cell suspension contained no measurable antibody.

These findings were confirmed in other experiments in which the donor rats were passively immunized with anti-sheep erythrocyte serum 24 hours prior to preparation of the spleen cells. In one experiment two spleen cell suspensions were prepared, one from normal donor rats and one from rats passively immunized with anti-sheep erythrocyte serum. After washing the volumes of the 2 suspensions were adjusted to contain 1×10^8 nucleated spleen cells per ml. Ten X-irradiated rats each received the spleen cell suspension from the normal rats, and 10 X-irradiated rats received the suspension from the passively immunized donor rats. Next day, 5 X-irradiated rats in each group were actively immunized with 0.5 per cent sheep erythrocytes, and 5 X-irradiated rats in each group with 10 μg of flagella. The 6 day titers, (Exp. 3 Table 4) showed that X-irradiated rats which received spleen cells from normal rats responded to sheep erythrocytes with moderately high titers, while rats which received cells from passively immunized donor rats had no or very low titers. The control X-irradiated rats which received one or the other spleen cell suspension and were immunized with flagella responded with equal agglutinin titers to S. typhosa; i.e., 4.8 for rats receiving normal spleen cells, and 4.0 for rats receiving cells from animals passively immunized with anti-sheep erythrocyte serum. Thus, the 2 cell suspensions had an equal capacity to respond to an antigen other than sheep erythrocytes.

These experiments demonstrated that specific antibody, undoubtedly bound to cells, effectively suppressed antibody formation. The 24 hours that elapsed between cell transfer and active immunization (Exp. 2 and 3, Table 4) should have been sufficient for any exchange or equilibrium to occur between passively given antibody bound to spleen cells and the circulating proteins of the X-irradiated recipients. Thus, a small amount of antibody, apparently bound onto or into potential antibody forming cells markedly suppressed the responsiveness of these cells to sheep erythrocytes.

In the following experiments the effect of passive immunization on spleen cells from previously immunized rats was measured.

The effect of passive immunization on the secondary antibody response of spleen cells

transferred to X-irradiated rats. Spleen cells were obtained from donor rats that had been actively immunized with 0.25 per cent sheep erythrocytes 4 weeks previously; the donor rats had very low circulating titers at the time of sacrifice. One x 10^8 nucleated spleen cells ("immune spleen cells") were injected into each of 10 rats 4 hours after X-irradiation. The following day, 5 of the X-irradiated rats were passively immunized with the rat anti-sheep erythrocyte serum, and 8 hours later all 10 rats were actively immunized with 0.5 per cent sheep erythrocytes. X-irradiated controls included rats which received spleen cells but were not actively immunized, and rats that were actively immunized only. Non X-irradiated control rats included normal rats that were passively immunized before active immunization, and normal rats actively immunized only. Table 5 shows that the X-irradiated rats which received "immune spleen cells" and were actively immunized had a mean titer of 7; passive immunization reduced the mean titer to 5. The X-irradiated rats which received "immune spleen cells" alone or sheep cells alone had no measurable antibody response. The mean titer for the primary response of the non-irradiated normal controls was 5 and passive immunization abolished the primary response.

Table 5

THE EFFECT OF PASSIVE IMMUNIZATION ON THE SECONDARY ANTIBODY RESPONSE OF SPLEEN CELLS TRANSFERRED TO X-IRRADIATED RATS

	Spleen cells from previously immunized donors‡	Passive immunization§	Active immunization¶	6 day antibody-titers*
X-irradiated rats	+	0	+	7.0 ± 1.08
	+	+	+	5.0 ± 0.55
	+	0	0	0 ± 0
	0	0	+	0 ± 0
Normal rats	0	0	+	4.8 ± 0.20
	0	+	+	0 ± 0

*Mean titers for groups of 5 rats ± the standard error of the mean.

†Rats each received 600 R total body X-irradiation.

‡Donor rats actively immunized with 1.0 ml of 0.25% suspension of sheep erythrocytes 4 weeks before sacrifice.

§Each passively immunized rat injected with 1.0 ml of anti-sheep erythrocyte serum 8 hours before active immunization.

¶Each immunized rat injected with 1.0 ml of 0.5% suspension of sheep erythrocytes.

Two findings were striking: First, the antibody response to spleen cells from previously immunized rats was considerably higher than the antibody response to spleen cells from normal rats in the previous experiments. Second, passive immunization produced much less suppression of the secondary response. These findings were confirmed.

Suppression of the antibody response by primary active immunization with small doses of antigen. The above experiments showed that an initial antigenic stimulation in the absence of antibody, apparently produced a population of cells capable of responding to antigen in the pres-

ence of antibody. However, antibody, circulating or bound to cells, suppressed the primary antibody response. Therefore, it seemed likely that after primary immunization, for as long as antibody was produced, subsequent response to injections of the same antigen might be limited to the population of cells originally specifically stimulated, or to the progeny of such cells. The production of antibody by these cells might suppress the response of potential antibody forming cells which were not stimulated during the first immunization, or which matured after the first immunization. In this manner, primary immunization with a critical small dose of antigen, resulting in the formation of a small amount of antibody, might suppress antibody formation to subsequent injections of antigen.

An immunizing dose of 0.25 per cent sheep erythrocytes, containing about 5×10^7 erythrocytes, regularly produced a moderately high 6 day antibody response in normal rats. Rats were injected intravenously with a suspension containing 5×10^7 sheep erythrocytes or with various dilutions of the same suspension. Some rats receiving a dose of 1×10^7 sheep erythrocytes responded with as high titers as rats that received a dose of 5×10^7 sheep erythrocytes; other rats receiving 1×10^7 sheep erythrocytes responded with very low or no measurable titers. Invariably, rats which received 5×10^6 or fewer sheep erythrocytes responded with no measurable titers. Rats receiving the various doses of sheep erythrocytes were re-injected with 5×10^7 sheep erythrocytes 4 or 6 days after the primary immunization and were bled for titers 6 days after the second immunization. Rats initially injected with 5×10^7 or with very low doses of less than 1×10^5 sheep erythrocytes responded with high titers to the second injection of 5×10^7 sheep erythrocytes. Some rats receiving an initial injection of 5×10^6, 1×10^6 or 5×10^5 sheep erythrocytes responded with moderately high titers to the second injection of 5×10^7 sheep erythrocytes while other rats receiving the same initial immunizations showed marked suppression of the antibody response to the second injection of 5×10^7 sheep erythrocytes. It was not possible to select a single initial immunizing dose of sheep erythrocytes which suppressed in all rats the response to a second injection of 5×10^7 sheep erythrocytes.

Suppression was sustained in some rats following multiple injections of antigen. Titers for individual rats are recorded in Table 6. Thus, suppression was produced by an initial active immunization with a small dose of antigen, but suppression was not obtained as regularly as by passive immunization prior to the first active immunization.

Plaque-forming cells and antibody response produced by small doses of antigens. Rats injected with small doses of 5×10^6 or fewer sheep erythrocytes had no measurable circulating antibody and showed a variable response to a second larger dose of sheep erythrocytes. A more sensitive method for measuring the immunological response to low doses of sheep erythrocytes might indicate the reason for this variability in the secondary response. The number of antibody producing cells in spleens of mice injected with large numbers of sheep erythrocytes correlated with antibody titer.[5,6] Similar results were obtained in rats; the highest numbers of plaque-forming cells in spleens of rats injected with 5×10^7 or more sheep erythrocytes were found 4 to 6 days after immunization.[9] This technique provided a possible means for determining the immunological response produced by low doses of antigen. Also, hemolytic antibody in low concentrations might be demonstrated on agar plates containing sheep erythrocytes.

Six rats were injected with a suspension containing 5×10^7 sheep erythrocytes; 6 rats were injected with a suspension containing 5×10^6 sheep erythrocytes, and 6 rats with a suspension containing 5×10^5 sheep erythrocytes. Six uninjected rats served as controls. Spleens and blood

Table 6

SUPPRESSION OF THE ANTIBODY RESPONSE BY PRIMARY ACTIVE IMMUNIZATION WITH SMALL DOSES OF ANTIGEN

Rat number	1st immunization*	6 Days		12 Days		18 Days		24 Days
		Antibody titer	2nd immunization	Antibody titer	3d immunization	Antibody titer	4th immunization	Antibody titer
1	5 X 10^7SRBC	5	5 X 10^7SRBC	5	5 X 10^7SRBC	7	5 X 10^7SRBC	6
2	5 X 10^7SRBC	5	5 X 10^7SRBC	5	5 X 10^7SRBC	7	5 X 10^7SRBC	7
3	5 X 10^7SRBC	5	5 X 10^7SRBC	5	5 X 10^7SRBC	7	5 X 10^7SRBC	6
4	5 X 10^7SRBC	7	5 X 10^7SRBC	6	5 X 10^7SRBC	7	5 X 10^7SRBC	6
5	5 X 10^7SRBC	5	5 X 10^7SRBC	6	5 X 10^7SRBC	7	5 X 10^7SRBC	7
6	5 X 10^6SRBC	0	5 X 10^7SRBC	7	5 X 10^7SRBC	6	5 X 10^7SRBC	6
7	5 X 10^6SRBC	0	5 X 10^7SRBC	3	5 X 10^7SRBC	3	5 X 10^7SRBC	5
8	5 X 10^6SRBC	0	5 X 10^7SRBC	3	5 X 10^7SRBC	3	5 X 10^7SRBC	4
9	1 X 10^6SRBC	0	5 X 10^7SRBC	4	5 X 10^7SRBC	3	5 X 10^7SRBC	3
10	1 X 10^6SRBC	0	5 X 10^7SRBC	5	5 X 10^7SRBC	4	5 X 10^7SRBC	4
11	5 X 10^6SRBC	0	5 X 10^7SRBC	0	5 X 10^7SRBC	0	5 X 10^7SRBC	0
12	5 X 10^6SRBC	0	5 X 10^7SRBC	0	5 X 10^7SRBC	0	5 X 10^7SRBC	0
13	1 X 10^6SRBC	0	5 X 10^7SRBC	0	5 X 10^7SRBC	0	5 X 10^7SRBC	3
14	1 X 10^6SRBC	0	5 X 10^7SRBC	0	5 X 10^7SRBC	0	5 X 10^7SRBC	0
15	1 X 10^6SRBC	0	5 X 10^7SRBC	0	5 X 10^7SRBC	0	5 X 10^7SRBC	0

*Rats receiving 5 X 10^6 or 1 X 10^6 sheep erythrocytes on the first immunization were arbitrarily separated into 2 groups and numbered; one group showed no suppression and one group showed marked suppression of antibody titers to subsequent injections of 5 X 10^7 sheep erythrocytes.

were obtained from 3 rats in each group 4 and 6 days after immunization. Numbers of plaque-forming cells in spleens, and serum hemolysin titers demonstrated on agar plates, are recorded for individual rats in Table 7.

Table 7

PLAQUE FORMING CELLS AND ANTIBODY RESPONSE PRODUCED BY SMALL DOSES OF ANTIGEN

Rat number*	Immunization†	Days after antigen injection	Plaque forming cells per spleen‡	Plaque forming cells per 10^6 spleen cells	Plate hemolysin titers	
					Whole serum	Highest serum dilution producing hemolysis
1	5×10^7SRBC	4	93,000	111	4+	128
2			55,500	65	4+	64
3			3,800	5	1+	whole
4		6	233,000	238	4+	512
5			148,000	214	4+	512
6			32,500	38	4+	128
7	5×10^6SRBC	4	2,500	3	0	
8			1,000	1	0	
9			150	0.2	0	
10		6	2,700	3	0	
11			2,000	2	0	
12			400	0.6	0	
13	5×10^5SRBC	4	1,500	2	0	
14			1,000	1	0	
15			350	0.5	0	
16		6	600	0.7	0	
17			450	0.6	0	
18			450	0.4	0	
19	None	4	83	0.2	0	
20			46	0.05	0	
21			38	0.04	0	
22		6	16	0.03	0	
23			28	0.03	0	
24			47	0.02	0	

*Rats arbitrarily numbered.

†Rats each injected intravenously with 1.0 ml of suspension containing 5×10^7, 5×10^6 or 5×10^5 sheep erythrocytes.

‡The total numbers of nucleated cells recovered from spleens varied from 4.9 to 10.2×10^8 cells per spleen.

The plaque-forming cell response varied considerably for rats receiving the same dose of antigen and sacrificed at the same interval after immunization. However, 5 to 238 plaque-forming cells per 10^6 recovered spleen cells were found for rats receiving the highest dose of antigen; sera from these rats contained antibody and titers for individual rats correlated with the number of plaque-forming cells obtained from their spleens. Rats injected with the 2 lower doses of antigen had increased numbers of plaque-forming cells in their spleens, although none had more than 3 plaque-forming cells per 10^6 recovered spleen cells. No antibody was demonstrated in any of the sera of the rats receiving the 2 lower doses of antigen. Individual rats, receiving either 5×10^6 or 5×10^5 sheep erythrocytes and sacrificed at the same time, had equivalent numbers of plaque-forming cells in their spleens. The experiment was adequate to demonstrate an immunological response to antigen in the absence of measurable serum antibody. The variable plaque-forming cell responses to the lower doses of antigen correlated with the variable suppression of the secondary response observed in the previous experiment.

DISCUSSION

It seems desirable to separate cells that are capable of responding to an antigen into 2 categories: "antibody forming cells" and "potential antibody forming cells." This separation has been based on reported differences between the primary and secondary responses. For example, the secondary response is less sensitive to inhibition by X-irradiation than the primary response.[10-12] Furthermore, the stimulation of antibody formation by addition of antigen to cells from previously immunized animals cultured in vitro,[13,14] or transferred to immunologically unresponsive recipients[15] is more easily achieved and with greater yield of antibody than when cells from normal animals are used. Recently it has been reported that normal cells are less sensitive to inhibition of antibody formation by actinomycin D than are cells from previously immunized animals.[16]

The present studies emphasize another difference. Clearly, immunization produces cells capable of responding to antigen in the presence of antibody. Four findings supported this conclusion. A secondary response was elicited when circulating antibody was present at the time of re-immunization.[1] Passive immunization did not inhibit the secondary response.[1] Antigen-antibody complexes formed in vitro elicited a high secondary response. Cells from previously immunized animals transferred to animals made unresponsive by X-irradiation responded to antigen in the presence of passively given antibody. In contrast, the antibody response of non-immunized animals or of cells from such animals was markedly inhibited by the presence of antibody. These findings supported this conclusion. The primary response was markedly inhibited by passive immunization. Antigen-antibody complexes formed in vitro elicited no measurable primary antibody response. Exposure of cells to antibody in non-immunized donors or in vitro suppressed their response to the antigen in X-irradiated recipients.

For the purposes of this discussion "antibody forming cells" are cells that have been modified by previous encounter with the antigen or possibly a closely related antigen. This does not exclude the possibility that some antibody forming cells may be "genetically determined" in their capacity to respond to the antigen. Antibody forming cells are cells that can respond to the antigen in the presence of specific antibody to the antigen. "Potential antibody forming cells" are cells from normal animals which at a given time have the capability of responding to a specific antigen, and are unresponsive to the antigen in the presence of specific antibody to the antigen. The use of the

terms, antibody forming cells and potential antibody forming cells, does not eliminate the possibility that more than one functional cell type may be included in one or the other designation.

It is generally accepted that antigen stimulates cell mitosis as an essential part of the early phase of antibody production. Hyperplasia of lymphoid tissue with increased numbers of pyroninophilic cells, indicating increased RNA synthesis, is a manifestation of this phase of the response. The inhibition of splenic hyperplasia by passive immunization provides indirect evidence that antibody prevents the initial stimulus of antigen to mitosis rather than the release of antibody from stimulated cells. Passive immunization also suppresses the marked increase in plaque forming cells in spleens of immunized rats,[9] thus adding further support to the findings reported here.

Although formation of antigen-antibody complexes in the circulation may prevent stimulation of potential antibody forming cells by the antigen, two findings strongly suggest that antibody adsorbed onto or into potential antibody forming cells suppresses their response to antigen. Normal spleen cells exposed to antibody in donor rats or in vitro and then washed free of excess antibody save a suppressed response to the antigen. Active immunization with small doses of antigen which produced no detectable circulating antibody produced marked suppression of the secondary antibody response in some rats. Our experiments provide no information on the mechanism whereby antibody prevents the stimulation of potential antibody forming cells by antigen. Presumably, the site of inhibition could be either on the cell surface or inside the potential antibody forming cell.

The variable suppression of the secondary response produced by initial immunization with small doses of antigen agreed with the variable numbers of plaque forming cells in the spleens. Sampling of the spleen alone for plaque forming cells was probably justified since splenectomy markedly decreased the antibody response of rats given small intravenous doses of sheep erythrocytes.[8] Furthermore, plaque forming cells were not found in lymph nodes of intact rats injected intravenously with small doses of sheep erythrocytes although a few occurred in lymph nodes of rats injected intravenously with the very large dose of 10^{10} sheep erythrocytes.[9] The failure to demonstrate circulating antibody in the presence of increased numbers of plaque forming cells in rats receiving low doses of antigen could be due either to the insensitivity of the system for measuring antibody or to uptake of antibody by potential antibody forming cells and possibly other cells. In the latter case circulating antibody would not reach appreciable levels until the cells taking up antibody were saturated.

The failure of a primary immunization to suppress the secondary response to a larger dose of antigen could occur in rats which had either a maximal or a minimal response to the small dose of antigen. Suppression presumably occurred when the initial antigenic stimulation was just sufficient to cause a balance between the number of antibody forming cells and the amount of antibody produced. Sufficient antibody had to be produced to block potential antibody forming cells from responding to the second injection of antigen; on the other hand, the numbers of antibody forming cells resulting from the initial immunization could not be sufficient to produce appreciably circulating antibody to the second injection of antigen.

In our experiments normal rats were unresponsive to antigen-antibody complexes formed in vitro; however, other antigen-antibody complexes may stimulate a primary response.[17] The capacity of complexes to stimulate a primary response might be due to adjuvant-like activity of the antigen of the complexes, or because of dissociation of the complexes. On the other hand, an

apparent primary response to antigen-antibody complexes could occur in animals in which there was some critical, but relatively small number of antibody forming cells to the antigen (or a closely related antigen) of the complexes.

It has recently been reported that X-irradiated mice injected with normal mouse lymphoid cells incubated in vitro with mouse anti-Salmonella adelaide serum and then immunized with the S. adelaide antigen responded with as high titers as mice receiving untreated lymphoid cells.[18] The difference between these findings and our findings is not understood. Possibly the Salmonella antigen contained endotoxin or other components which have an adjuvant like action not possessed by sheep erythrocytes.

Immunological unresponsiveness has been produced in adult animals by various means. The suppression of the primary antibody response by passive immunization has been repeatedly observed in various species and with various bacterial, viral and cellular antigens, suggesting that the phenomenon is widespread. These findings and the possible relationship between this phenomenon and immunologic enhancement of tumor or skin homografts has been discussed.[1,18,19] Both delayed hypersensitivity and circulating antibody production were suppressed in mature guinea pigs by feeding of a hapten[20] or by injecting very small amounts of the hapten or a soluble protein into mesenteric veins.[21,22] Unresponsiveness to heterologous serum proteins was induced in adult mice by initial injections of small quantities of soluble protein cleared of particulate matter having adjuvant like activity. Partial unresponsiveness developed in some mice within 3 days after the initial injection of protein, and unresponsiveness developed regularly when the interval was 5 to 12 days between injections.[23,24]

The fact that adult animals of different species can be made unresponsive to various antigens either by passive immunization or by exposure to small amounts of antigen would seem to indicate a common underlying principle. Our experiments suggest that antibody, either passively administered or actively produced in small quantities, may limit production of specific antibody to that portion of the antibody forming system previously stimulated by the antigen. Thus, low levels of antibody formation may be responsible for maintaining immunological unresponsiveness. This suggestion is strengthened by the demonstration of antibody forming cells in the absence of detectable circulating antibody. This mechanism may account in part for immunological unresponsiveness produced in certain other related experimental systems.

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BIOLOGIC PROPERTIES OF POLYNUCLEOTIDES
V. STUDIES ON THE INHIBITION OF THE FIRST COMPONENT OF COMPLEMENT BY POLYINOSINIC ACID; THE INTERACTION WITH C'1q*

By

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Recent reports from this laboratory[1,2] have revealed that poly I‡ is an inhibitor of the first component of C' (C'1), but has no effect upon C'1 esterase activity.[3] This phenomenon led to the speculation that poly I might inhibit C'1 by interacting with it at a molecular site which did not affect esterase activity per se, but rather some other functional portion of the molecule. Subsequent studies (by other investigators) described the resolution of C'1 activity into three separate protein components, C'1q, C'1r, and C'1s, all three of which were necessary for C'1 activity. C'1q was shown to be identical with the 11S component; C'1s was identified as C'1 proesterase; C'1r was a component of C'1 never before described.[4] It was further shown that these subcomponents comprise a macromolecular complex which is responsible for hemolytic C'1 activity.[5] Experiments were therefore designed to see whether poly I could be shown to interact specifically with one or more of the subcomponents of C'1. Our results demonstrate that poly I is a specific inhibitor of the C'1q portion of the C'1 macromolecule. In addition, the inhibition of C'1 hemolytic activity was studied in greater detail than was previously possible.

METHODS AND MATERIALS

Synthetic polynucleotides. Poly A, poly I and poly C were synthesized as previously described;[1] poly G was synthesized by means of RNA polymerase utilizing a poly C template.[2]

Buffered saline diluents. Both triethanolamine buffered saline (TBS) and barbital buffered saline (BBS), pH 7.4, ionic strength 0.15, were used. The usual divalent cation concentrations were 1.5×10^{-4} M Ca^{++} and 5×10^{-4} M Mg^{++}. For the dilution of C'1q, C'1r, and C'1s and the preparation of EAC'_1 by means of these reagents buffered saline containing only 1×10^{-3} M Ca^{++} was employed. Buffered saline containing 2.6×10^{-2} M Na_2Mg EDTA was used as diluent when studying the lysis of EAC'_1 in either guinea pig or human complement dilutions, the final concentration of Na_2Mg EDTA being 1.6×10^{-2} M. Buffered saline containing Na_3HEDTA was employed in certain experiments.

Complement reagents. Pooled human serum and guinea pig serum were stored in aliquots at -40° or -60° C. C'1q, C'1r, and C'1s were prepared by DEAE chromatography of a human serum euglobulin fraction as described by Lepow et al.[4] Fractions containing peak activity of each constituent were pooled and frozen in aliquots at -60° C. Before use, the fractions were diluted

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‡The following abbreviations are used: poly I, poly A, poly G, poly C, poly U; the synthetic homoribopolynucleotides of inosinic, adenylic, guanylic, cytidylic, and uridylic acids respectively: poly GU; a mixed copolymer of guanylic and uridylic acids: poly IU; a mixed copolymer of inosinic and uridylic acids.

in buffered saline containing 1 x 10^{-3} Ca^{++}. Diluted fractions were stored at 4° C for as long as 1 month with no detectable loss of activity.

Sensitization of erythrocytes. Sheep erythrocytes were sensitized either with Forssman antiserum or with commercially obtained glycerinated amboceptor* as previously described.[4] By these methods, lysis of EA exposed to C'1r and C'1s (without C'1q) in human or guinea pig serum diluted in Na_2Mg EDTA buffered saline was always negligible (OD 540 < 0.030). Complete hemolysis of 1.0 ml (5 x 10^8) EA in a volume of 4.0 ml yields an O.D. at 540 mμ of approximately 1.31.

Preparation of EAC'_1. Five-tenths ml each of an appropriate dilution (usually 1:100) of stock C'1q, C'1r and C'1s were added to a centrifuged button of 5 x 10^8 EA at 37° and incubated for 10 minutes. In certain experiments whole C'1 was generated by allowing equal volumes of C'1q, C'1r, and C'1s to incubate at 37° for 5 to 10 minutes. EAC'_1 were then formed by adding 1.5 ml of this C'1 to a button of 5 x 10^8 EA. If the cells were to be washed or otherwise treated before lysis, they were kept at 37° to avoid the loss of activity which occurs at lower temperatures with $EA_{hu}C'_1$.[4] A washed button of 5 x 10^8 EAC'_1 was lysed in 4 ml of a 1:100 dilution of human serum in buffered saline containing 1.6 x 10^{-3} M Na_2Mg EDTA. If no washing was required, 2.5 ml of a 1:62.5 dilution of human serum in buffered saline containing 2.6 x 10^{-2} M Na_2Mg EDTA was added to the tube. $EA_{hu}C'_1$ could be lysed in Na_2Mg EDTA guinea pig serum and their behavior then paralleled in every way their behavior in the human serum reagent. When guinea pig serum was used in the lytic step, 2.5 ml of a 1:100 dilution (2.6 x 10^{-2} M Na_2Mg EDTA BBS) was added to 1.5 ml $EA_{hu}C'_1$ containing 5 x 10^8 EA. Hemolysis was carried out at 32° C for 60 minutes and the extent of lysis was determined by reading the optical density of the supernatant solution at 540 mμ. An EA blank was always included.

Density gradient ultracentrifugation. Generation of C'1 activity from C'1q, C'1r and C'1s involves the recombination of the three subcomponents of C'1 into a macromolecular complex having whole C'1 activity. If a mixture of the three C'1 subcomponents is subjected to density gradient ultracentrifugation in a sucrose gradient containing 10^{-3} M Na_3 HEDTA, no recombination occurs and C'1q, C'1r, and C'1s activity can be recovered as three separate peaks. In the absence of EDTA, however, whole C'1 activity can be found at a density level consistent with a molecular size greater than any of the subcomponent activities.[5] The general procedure was as follows: 10 to 40 per cent sucrose density gradients were prepared in an appropriate TBS: an aliquot of the C'1q, C'1r, C'1s mixture was layered on top; the preparation was then centrifuged in the SW-39 rotor of the Spinco Model L preparative ultracentrifuge (35,000 RPM) for 16 hours at 6° C. Three-drop fractions were then collected by needle puncture of the bottom of the tube into 4.0 ml 0.15 M NaCl, and all fractions were dialyzed against TBS (10^{-3} M Ca^{++}) for 16 hours at 4° C. Each fraction was then tested for C'1q, C'1r, C'1s and whole C'1 activity by adding 0.5 ml of the test fraction to a button of EA (5 x 10^8) together with 0.5 ml of a 1:100 dilution of the other two components. After incubation at 37° for 10 minutes, 2.5 ml of a human serum dilution, containing 2.6 x 10^{-2} M Na_2Mg EDTA was added and hemolysis carried out. Whole C'1 activity was tested similarly save that 1.5 ml of the fraction in question was added (without subcomponents) to an EA button. A simultaneously run gradient tube containing bovine gamma globulin (7S) and porcine thyroglobulin (19S) as markers was used to assess approximate molecular size.[5]

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EXPERIMENTAL AND RESULTS

The inhibition of purified C'1 by poly I. Whole C'1 was formed by incubating equal volumes of C'1q, C'1r, and C'1s (1:100 dilutions) at 37° for 10 minutes. Two ml aliquots of this human C'1 were added to tubes containing 0.1 ml poly I solution of varying concentrations. Following a further 2-minute incubation at 37°, 1.5 ml of each poly I-C'1 mixture was added to a button of 5×10^8 EA and incubated for another 10 minutes at 37°. Two and five-tenths ml of a 1:100 Na_2Mg EDTA-BBS guinea pig serum dilution was added to each tube and the degree of lysis measured. Control EAC'_1 cells (formed in the absence of poly I) were tested for lysis in Na_2Mg EDTA guinea pig serum to which poly I in equivalent amounts had been added.

This experiment (Table 1) demonstrates that detectable inhibition of EAC'_1 formation occurs with less than 2×10^{-4} μmol P poly I per ml C'1; 1×10^{-3} μmol P poly I per ml is completely inhibitory. The amount of poly I needed to inhibit EAC'_1 formation is very much less than that required to inhibit the lysis of the intermediate complex EAC'_1 when pre-added to Na_2Mg EDTA-BBS diluted guinea pig serum, where as much as 1 μmol P poly I still allowed perceptible lysis to occur (O.D. 540 equals 0.140).

Table 1

THE INHIBITION OF PURIFIED C'1 BY POLY I

μmol P poly I added	Reagent to which poly I is added	
	C'1	Na_2Mg EDTA guinea pig serum (1:100 dilution)
	O.D.*	O.D.*
0	1.305	1.245
0.000032	1.295	N.D.†
0.000063	1.284	N.D.†
0.000125	1.250	N.D.†
0.00025	0.860	N.D.†
0.0005	0.288	1.230
0.001	0.022	1.230
0.002	0.000	1.232
0.004	0.000	1.277
0.01	0.000	1.216
1.0	N.D.†	0.140

*Optical density at 540 mμ, corrected for EA control.

†Not done.

Earlier work has shown that poly I incorporated into hydrogen-bonded helical structures with poly A or polycytidylic acid (poly C) is incapable of inhibiting whole C' activity.[1] The following experiment was performed to test the effect of a poly I-poly A* double-stranded helix on human C'1 activity.

*In most of the experiments involving poly I and poly A helical complexes the molar ratio of poly A:poly I was kept at 1.5:1. Under these circumstances, a 1:1 complex is formed, hereinafter designated as poly (I + A). The triple-stranded helix (2I + A) is formed only in the presence of an excess of poly I.

One-tenth ml undiluted C'1q, C'1r, and C'1s were added to 9.7 ml TBS containing 10^{-3} M Ca^{++} and incubated at 37° for 5 minutes to generate C'1. One ml poly I (0.02 μmol P per ml) was added to 1.0 ml C'1 and held at 25° for 15 minutes. One ml poly A (0.03 μmol P per ml) was then added and the mixture held at 25° for another 5 minutes. Other 1 ml C'1 aliquots were treated in similar fashion with 1.0 ml additions containing: TBS followed by poly A, TBS followed by poly (I + A), and TBS followed by TBS (the actual amounts of poly I and poly A added were constant in all tubes). One and five-tenths ml of each of the four C'1 preparations were then tested for their capacity to form EAC'_1.

Under these conditions, the addition of Poly A to C'1 exposed to poly I does not restore C'1 activity (Table 2). Presumably all excess poly I is bound to poly A and is therefore incapable of exerting further inhibition, since poly (I + A) (preformed) has no capacity to inhibit C'1 activity. Poly A is again used as a "stopping" reagent in the study of the effects of poly I on the C'1 subcomponents (vide infra).

Table 2

THE EFFECT OF VARIOUS POLYNUCLEOTIDE ADDITIONS ON HUMAN C'1

	Polynucleotide added			
	poly I + poly A	poly A	poly (I + A)	None (control)
Hemolysis* (O.D. 540)	0.000	0.326	0.354	0.362

*Lysis performed in Na_2Mg EDTA human serum.

The inhibition of $EA_{hu}C'_1$ by poly I. The capacity of poly I to inhibit $EA_{g.p.}C'_1$ has previously been demonstrated.[3] The sensitivity of $EA_{hu}C'_1$ to poly I was studied utilizing the purified human C'1 material.

To the centrifuged cell button from 50 ml EA were added 25 ml each of 1:100 C'1q, C'1r, and C'1s. Following a 10-minute incubation at 37°, 125 ml TBS were added. The cells were centrifuged, and were resuspended in 100 ml TBS. Two ml aliquots of EAC'_1 were added to individual tubes. These were centrifuged and the supernatant fluid discarded. The cell button was then resuspended in 1 ml TBS containing varying amounts of poly I and incubated at 37° for 10 minutes. Following this the cells were washed twice with 4.0 ml volumes of TBS, resuspended in 4.0 ml of a 1:100 dilution of human serum in 1.6×10^{-2} M Na_2Mg EDTA TBS, and incubated at 32° for 1 hour.

The incubation of $EA_{hu}C'_1$ with as little as 8×10^{-4} μmol P poly I is capable of inhibiting their subsequent lysis by > 90 per cent (Table 3).

The inactivation of C'1q by poly I. Since poly I was found to be such a potent inhibitor of whole C'1 activity, any attempt to demonstrate selective inhibition of one of the C'1 subcomponents by poly I was dependent upon a method of neutralizing its Anti-C'1 properties subsequent to exposure to a given subcomponent, since the presence of free or "active" poly I during the

Table 3

THE INHIBITION OF $EA_{hu}C'_1$ BY POLY I

μmol P poly I exposed to $EA_{hu}C'_1$	O.D. 540 mμ
0.1	0.009
0.05	0.013
0.025	0.010
0.0125	0.014
0.00625	0.016
0.0031	0.024
0.0016	0.038
0.0008	0.068
0	0.876

phase of whole C'1 generation would simply result in whole C'1 inhibition. Since poly A added to a poly I-treated C'1 preparation did not restore whole C'1 activity, and since poly (I + A) did not inhibit C'1 activity, subcomponents of C'1 were exposed to poly I; poly A was then added as a "stopping" reagent to prevent inhibition of subsequently generated whole C'1 activity, and following addition of the other two untreated subcomponents, the capacity of the mixture to form EAC'_1 was measured.

Aliquots of C'1q, C'1r, and C'1s were added to a given amount of poly I. Following 2' incubation at 37° a 50 per cent molar excess of poly A was added to each and incubation was continued at 37° for another 2 minutes. The mixtures were then tested immediately, or in some instances, placed on ice. Aliquots of each component were exposed under the same conditions to: saline followed by poly A; equivalent amounts of poly (I + A); poly I followed by saline. Control subcomponents diluted with equivalent amounts of saline were incubated at 37° for 4 minutes. Five-tenths ml of each subcomponent treated with a given polynucleotide combination was then added to a button of 5×10^8 EA; 0.5 ml of the other two control (saline-treated) subcomponents were added, and after 10 minutes at 37°, the cells were tested for EAC'_1 formation.

The results presented in Table 4 reveal several interesting points, of which the most striking is the selective inhibition by poly I (followed by poly A) of C'1q. As expected, poly I alone (used here in excess on the basis of whole C'1 inhibition) added to any one of the 3 subcomponents resulted in total inhibition of C'1 generation. Poly A alone resulted in partial inhibition of lysis (3 of 4 experiments) regardless of the component to which it was added. Poly (I + A) was non-inhibitory. Poly I followed by poly A had a selective inhibitory effect on C'1q and resulted in > 95 per cent inhibition of C'1 generation in 3 of 4 experiments. Poly I followed by poly A was least effective in that experiment which utilized the largest amount of the two homopolymers. This phenomenon will be commented on further.

The experiment shown in Figure 1 was performed to obtain an estimate of the minimum amount of poly I necessary to completely inactive C'1q when poly A is used as a "stopping" reagent. 2.5×10^{-3} μmol P poly I per ml 1:100 C'1q will completely inhibit C'1q activity under these conditions.

Since functional whole C'1 activity apparently resides in a macromolecule, the isolation of

Table 4

THE INHIBITION OF C'1q BY POLY I

Additions to C'1 subcomponent	C'1 subcomponent exposed	EAC'_1 formation (O.D. 540 mμ)			
		Experiment			
		I	II	III	IV
poly I, poly A	C'1s	0.595*	0.311*	1.314*	0.717*
	C'1r	0.605	0.299	1.304	0.657
	C'1q	0.000	0.013	0.057	0.213
saline, poly A	C'1s	0.345	0.171	1.299	0.483
	C'1r	0.431	0.181	1.298	0.442
	C'1q	0.552	0.207	1.284	0.437
saline, poly (I + A)	C'1s	0.613	0.285	1.317	0.700
	C'1r	0.756	0.288	1.299	0.660
	C'1q	0.763	0.335	1.299	0.684
poly I, saline	C'1s	N.D.	0.000	0.014	0.008
	C'1r	N.D.	0.000	0.009	0.007
	C'1q	N.D.	0.000	0.007	0.005
Omit	C'1s	0.002	0.004	N.D.	0.005
	C'1r	0.003	0.004	N.D.	0.006
	C'1q	0.027	0.010	N.D.	0.007
Control		0.983	0.385	1.315	0.720

*The following conditions were employed:

Exp. I: 1 ml 1:50 subcomponent dilution + 0.5 ml poly I (0.02 μmol P per ml) + 0.5 ml poly A (0.03 μmol P per ml). Lysis in Na_2Mg EDTA human serum.

Exp. II: 1 ml 1:100 subcomponent dilution + 0.5 ml poly I (0.01 μmol P per ml) + 0.5 ml poly A (0.015 μmol P per ml). On ice for approximately 1 hour prior to test. Lysis in Na_2Mg EDTA guinea pig serum.

Exp. III: 2 ml 1:100 subcomponent dilution + 0.1 ml poly I (0.1 μmol P per ml) + 0.1 ml poly A (0.15 μmol P per ml). On ice for approximately 1 hour prior to test. Lysis in Na_2Mg EDTA guinea pig serum.

Exp. IV: 3 ml 1:100 subcomponent dilution + 0.05 ml poly I (4 μmol P per ml) + 0.075 ml poly A (4 μmol P per ml). Lysis in Na_2Mg EDTA guinea pig serum.

the three subcomponents C'1q, C'1r and C'1s is in a strict sense an artifact mediated by the presence of sufficient EDTA to chelate Ca^{++}.[5] Readdition of Ca^{++} results in reaggregation of the subcomponents into a macromolecule with functional activity of C'1.[5] The possibility that poly I might function in a similar manner (that is, by breaking the native C'1 molecule into its 3 subcomponents) was tested by utilizing density gradient ultracentrifugation of C'1 to which poly I, followed by poly A, had been added. The results of this experiment (Figure 2) offer further proof of the specific inhibition of C'1q by poly I, and indicate that poly I does not inactivate C'1 by splitting it into its 3 subcomponent parts, but rather by rendering the entire molecule inactive (in terms of ability to form EAC'_1).

Five-tenths ml each of undiluted C'1q, C'1r and C'1s were mixed together and held at 32° C for 5 minutes to generate whole C'1 activity. 0.165 ml poly I (10 μmol P per ml) was added, a

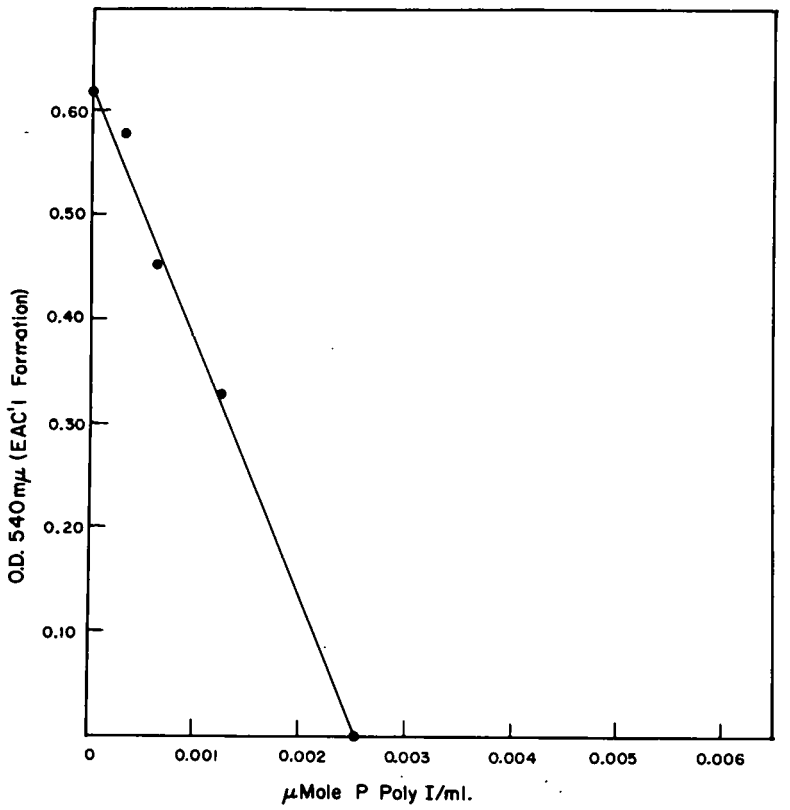

Figure 1. Titration of the inhibition of C'1q by poly I. C'1q (1.0 ml, 1:50 dilution) was mixed with 0.5 ml containing varying amounts of poly I. Following 2 minutes at 37°, 0.5 ml containing a 50% molar excess of poly A was added. 0.5 ml of each test were then added to 0.5 ml each C'1r and C'1s (1:100 dilution) and 5 x 10^8 EA and tested for C'1 forming ability.

faint precipitate appeared immediately, and incubation continued for another 5 minutes. 0.165 ml poly A (11.5 μmol P per ml) was added. Two minutes later two 0.8 ml aliquots were removed; to one was added sufficient Na_3 HEDTA (in 0.1 ml TBS) to give a final concentration of 1 x 10^{-3} M Na_3 HEDTA (gradient I). To the other was added 0.1 ml TBS (gradient II). After 5 minutes further incubation at 32° C, the two solutions were placed on ice. 0.15 ml of each of the above were layered over a 10 to 40 per cent sucrose density gradient made up in TBS with 10^{-3} M Ca^{++} (gradient II) or with 10^{-3} M Na_3 HEDTA (gradient I). A third gradient received the bovine gamma globulin-porcine thyroglobulin markers. All three tubes were then centrifuged and analyzed for protein (gradient III) and for whole C'1 and C'1 subcomponent activity (gradients I and II) as described earlier.

The aliquot which contained whole C'1, poly I, poly A, and Ca^{++} (gradient II) is not depicted in Figure 2 since no hemolytic activity could be demonstrated in the dialyzed fractions. The aliquot which contained all the above components, but which was subsequently chelated with Na_3 HEDTA (gradient I), shows C'1r and C'1s subcomponent activity, but no C'1q activity. Whole C'1 activity was not detected, but this may simply reflect the presence of Na_3 HEDTA, and is not necessarily caused by poly I.

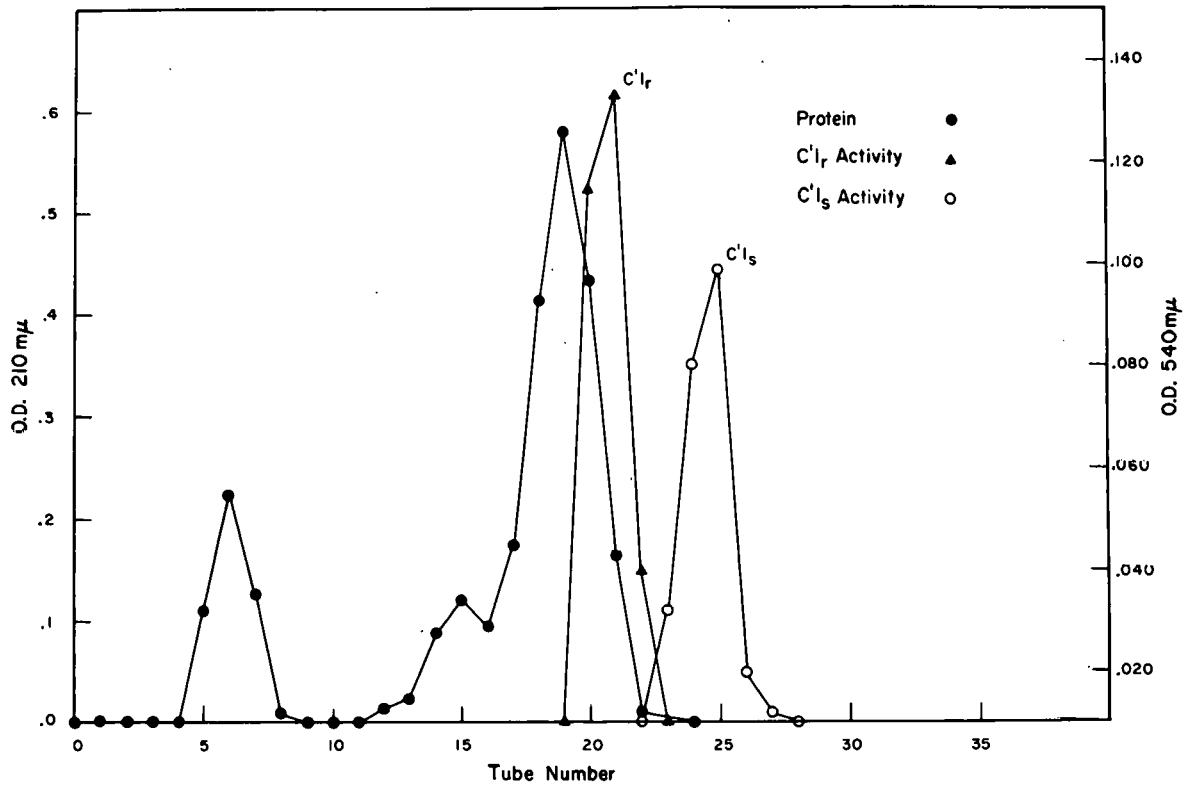

Figure 2. Density gradient ultracentrifugation of poly I treated C'1. Undiluted C'1q, C'1r and C'1s were mixed together in the presence of 1 x 10^{-3} M Ca^{++}. Following the sequential addition of poly I and poly A, Na_3 HEDTA was added to a final concentration of 1 x 10^{-3} M. An aliquot was subjected to sucrose density gradient ultracentrifugation and the collected fractions were analyzed for whole C'1 hemolytic activity and for C'1q, C'1r and C'1s subcomponent hemolytic activity (O.D. 540 mμ). Only C'1r and C'1s activity could be detected. A simultaneously run gradient tube containing porcine thyroglobulin (19S, left hand peak) and bovine gamma globulin (7S, right hand peak) was analyzed for protein content (O.D. 210 mμ).

The reversibility of the inactivation of C'1q and C'1 by poly I. It was mentioned above that when larger amounts of poly I were added to C'1q with subsequent addition of poly A, the inhibition of C'1q was consistently less efficient than when more critical amounts of each homopolymer were used. Since equivalent amounts of poly I alone always yielded complete inhibition, this suggested that the inactivation of C'1q by poly I was reversible by poly A. The following experiment was designed to test this hypothesis.

Five-tenths ml poly I (0.01 μmol P per ml) was added to 1 ml 1:100 dilution of C'1q. After 2 minutes incubation at 37°, 0.5 ml poly A (0.015 μmol P per ml) was added and incubation was continued for another 2 minutes. One ml aliquots C'1q were treated in a similar fashion with the following 0.5 ml addition: poly I followed by BBS, BBS followed by poly A, poly (I + A) followed by BBS, and BBS followed by BBS. Immediately after the 4 minute period 0.5 ml of each mixture was added to 5 x 10^8 EA, followed by 0.5 ml each C'1r and C'1s (1:100 dilution) and EAC'_1 formation was measured. The 5 mixtures were then kept at 4° C for 16 hours, and retested in a similar fashion.

Table 5 reveals that immediately after addition no C'1q activity is detectable in either frac-

Table 5

THE REVERSIBILITY OF POLY I INHIBITION OF C'1q ACTIVITY BY POLY A

Polynucleotide added to C'1q	C'1q activity (EAC'_1 formation)* O.D. 540 mμ	
	Immediately after incubation	16 hours at 4° C
poly I + Poly A	0.008	0.028
poly I	0.005	0.000
poly A	1.131	0.758
poly (I + A)	1.286	1.095
None (BBS control)	1.296	1.173

*Lysis in Na_2Mg EDTA guinea pig serum

tion to which free poly I was added. After 16 hours, however, significant C'1q activity has reappeared in the C'1q fraction where poly I was followed by a 50 per cent excess of poly A, whereas the C'1q which received only poly I is still devoid of C'1q activity. If larger amounts of poly I and poly A are sequentially added to a C'1q fraction, the return of C'1q activity is usually complete after 16 hours incubation at 4° C. A similar return of significant activity was noted when whole C'1 was treated with poly I, followed by poly A but restoration of whole C'1 activity was only partial.

The inhibition of human C'1 by other synthetic polynucleotides. The ability of other synthetic ribopolynucleotides to inhibit human C'1 activity was assessed by the method outlined earlier for poly I, with the results shown in Table 6. Their relative potency is similar to that previously noted for whole C' inhibition.[1,2]

DISCUSSION

These results confirm the speculation that poly I inhibits C'1 by interaction with the C'1 molecule at a site distinct from that directly responsible for C'1 esterase activity. Considering that the interaction of C'1q (or 11S component) is the earliest feasible step in the formation of intermediate complexes between C' and EA,[5-7] and considering the precipitable complexes it forms with 7S gamma globulin aggregates,[8,9] it is not unreasonable to suppose that the C'1q subcomponent (or portion of the C'1 molecule) does in fact represent the hypothetical attachment site[10] of the C'1 molecule to EA. Thus poly I inhibits the hemolytic properties of C'1 without reducing its esterolytic potency by preventing its attachment to EA. The inactivation of EAC'_1 is explained without difficulty since it is recognized that C'1 is not irreversibly fixed to the cell, but is, even in the absence of poly I, constantly eluting and reattaching;[11] in addition, poly I may in some way actually promote detachment. Conceptually such a mechanism of C'1 inactivation is distinctly different from that involving C'1 esterase inhibition where an inactivated EAC'_1 cell might still bear a C'1 molecule whose function was blocked, whereas EAC'_1 inactivated by poly I should bear no C'1 molecules, active or inactive, and all SA sites on such a cell should be freely available for attachment of fresh C'1 molecules. Such, in fact, is the case,

Table 6

THE CAPACITY OF VARIOUS SYNTHETIC POLYRIBONUCLEOTIDES TO INHIBIT HUMAN C'1

Polynucleotide	μmol P polynucleotide added	Reagent to which polynucleotide is added	
		C'1 O.D.*	Na_2Mg EDTA guinea pig serum O.D.*
I. Homopolymers			
Poly A	0.4	1.230	N.D.†
Poly C	0.4	1.230	N.D.
Poly U	0.4	1.235	N.D.
Poly G	0.1	0.065	1.276
Poly G	0.02	0.960	1.231
II. Poly GU			
85% G, 15% U	0.4	0.184	1.230
66% G, 34% U	0.4	1.227	N.D.
32% G, 68% U	0.4	1.237	N.D.
III. Poly IU			
83% I, 17% U	0.4	0.530	1.210
56% I, 44% U	0.4	1.250	N.D.
31% I, 69% U	0.4	1.200	N.D.
IV. Saline control		1.235	1.236

*Optical density at 540 mμ, corrected for EA control.

†Not done.

since experiments have shown that poly I-inactivated EAC'_1 are fully capable of complete EAC'_1 reformation if fresh C'1 is added.[12]

The density gradient ultracentrifugation experiments support the conclusion that poly I inactivates the entire C'1 molecule (in the presence of Ca^{++}) presumably at the C'1q portion of the macromolecule. This does not prevent the appearance of active C'1r and C'1s when EDTA is added subsequently. By use of the isolated C'1 subcomponents with poly A as a stopping reagent, it can be shown that poly I selectively inactivates C'1q. If we presume that this is effected by some sort of complex formation, two puzzling features can be understood. These phenomena are: 1) That C'1q inactivation is less efficient when larger amounts of poly I (and poly A) are used, and 2) That more than 10 times the amount of poly I is required than is necessary to inhibit completely an equivalent amount of C'1q in whole C'1 (where poly A addition is not necessary).

Recognizing that, given sufficient time, poly A addition can reverse C'1q and C'1 inhibition by poly I, the reaction mechanism can be simply depicted in the following expressions:

$$\text{Reaction I} \qquad \begin{matrix}\text{Reaction} \\ \text{II} \\ \text{poly I} \\ + \\ \text{poly A} \\ \downarrow \\ \text{poly (I + A)}\end{matrix} \quad + \quad \text{C'1q} \rightleftharpoons \text{poly I - C'1q complex}$$

Even with critical amounts of poly I and poly A (in terms of reaction I), reaction II is favored, and in time will slowly reverse reaction I. With larger amounts of poly I (and of necessity, poly A) the kinetics of a bimolecular reaction predict that the rate of poly (I + A) formation will increase geometrically, while the rate of C'1q - poly I complex formation can increase only in an arithmetic fashion since C'1q concentration remains constant. This means that poly A addition to a poly I - C'1q complex sets up a system in which two separate bimolecular reactions are competing for poly I. Increases in poly I and poly A concentration promote the rate and extent of poly (I + A) complex formation, which favors the dissociation of the preformed poly I - C'1q complex. Hence one may visualize how C'1q activity can be restored. In addition, at any given moment after poly A addition, only a fraction of the poly I present is actually available for interaction with C'1q, and this probably explains why greater poly I concentrations are needed to inhibit C'1q when studied in this fashion. Nevertheless, the poly I - C'1q - poly A system is useful, if only because it allows identification of C'1q as the susceptible component of C'1 activity.

The relative ability of the various synthetic ribopolynucleotides to inhibit C'1 activity parallels their rank order in the inhibition of whole C' activity.[1,2] In both these phenomena there is a striking decrease in activity when mixed IU copolymers are used; only the 83 per cent I, 17 per cent U polymer has any Anti-C'1 activity, and poly I is approximately 2000 times more effective in inhibiting C'1; indeed, the potency of poly I as a complement-inhibiting agent relative to the remaining polynucleotides, is much greater in the C'1 system than in the whole C' system. As in the whole C' system, the only other homopolymer capable of inhibiting C'1 is poly G (which has only 1/100 the inhibitory activity of poly I in the latter system).

Despite the apparent specificity of C'1 inhibition by poly I, attempts to reconstitute the hemolytic potency of poly I-treated serum by addition of C'1 or C'1 subcomponents have not met with success. The reasons for this failure are the subject of a subsequent report.

ACKNOWLEDGMENT

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BIOLOGIC PROPERTIES OF POLYNUCLEOTIDES. VI. FURTHER STUDIES ON THE MECHANISM OF COMPLEMENT INHIBITION BY POLYINOSINIC ACID: THE INACTIVATION OF THE FOURTH COMPONENT OF COMPLEMENT*

By

S. Yachnin,† D. Rosenblum, and D. Chatman

In the previous report it was noted that attempts to restore the hemolytic potency of poly I-inactivated serum by the addition of purified C'1 or C'1q met with no success.[1] This suggested that some other C' component might be inactivated during the interaction of poly I with whole serum C'. To explore this possibility, means were sought of studying the effects of poly I on C' components not bound to EA. A method was required which would allow poly I to act upon C', and would then be capable of neutralizing the Anti-C' properties of poly I, so that any changes in the activity of C' components not reversible by the method of neutralization could be subsequently studied. Earlier studies have shown that polyadenylic acid (poly A) or polycytidylic acid (poly C), when premixed with poly I, or when added to whole C' immediately after the addition of poly I, were capable of preventing poly I from acting as an Anti-C' agent.[2] Studies with purified C'1 and C'1q have shown that the incorporation of poly I into a poly (I + A)‡ double stranded helix nullifies the inhibitory action of poly I; and further, that the inhibition of C'1q and C'1 by poly I is at least partially reversible by the subsequent addition of poly A.[1] The present experiments were designed to ascertain whether these 2 homopolymers (poly A and poly C) (a) could function as "stopping" agents so as to remove poly I as an active Anti-C' agent from a whole C' system, (b) could totally or partially reverse the Anti-C' effects of poly I upon C' components in solution, and (c) (assuming lack of reversal, or only partial reversal) could allow the whole C' system, inactivated in some way by poly I but presently free of "active" poly I, to be studied in such a way as to reveal which component(s) of C' had been inactivated during exposure to "active" poly I. The results of these studies demonstrate that in addition to the (reversible) inactivation of C'1, poly I causes the irreversible inactivation of C'4.

MATERIALS AND METHODS

I. Synthetic Polynucleotides

Poly A, poly I, and poly C were synthesized as previously described.[1]

II. Complement Reagents

Guinea pig blood obtained by cardiac puncture was allowed to clot for 4 hours at room tem-

*This report is taken from a paper that appeared in J. Immunol., 93:549, 1964. The work was supported in part by the Helen and Joseph Regenstein Foundation.

†John and Mary Markle Scholar in Academic Medicine.

‡In all experiments described in this paper the formation of the poly (I + A) double stranded helix was insured by use of a molar excess (approximately 50 per cent) of poly A. Under conditions of poly I molar excess a triple stranded helical structure is formed (2I + A).

perature. The serum was then separated, pooled, and stored in small aliquots at -40°C. Pooled human serum was stored in similar fashion. Human C'1q, C'1r, and C'1s were prepared by DEAE cellulose chromatography of a human serum euglobulin fraction as described by Lepow et al.[3]* Whole human C'1 was prepared by mixing these 3 components at 37°C as previously described.[1] Guinea pig C'1 was prepared by the method of Becker.[4] As previously noted,[1] it was found that human C'1 could replace guinea pig C'1 in a guinea pig C' hemolytic system. Guinea pig C'4 was prepared by stepwise elution of a guinea pig serum euglobulin supernatant from DEAE cellulose as outlined by Becker.[5] R4 was prepared by published procedures.[6] Human C'1 esterase was prepared by the method of Haines and Lepow, and contained 188 units per ml.[7,8]

Buffered saline diluents. Barbital buffered saline (BBS) pH 7.4, ionic strength 0.15, containing 1.5×10^{-4} M Ca^{++} and 5×10^{-4} M Mg^{++} was the standard diluent. C'1 and C'1 subcomponents were usually diluted in BBS containing only 1×10^{-3} M Ca^{++}. Na_3 HEDTA-BBS contained 7.5×10^{-3} M Na_3 HEDTA; Na_2Mg EDTA-BBS contained 15×10^{-3} M Na_2Mg EDTA.

Sensitization of erythrocytes. Commercial glycerinated amboceptor† was used throughout. Red cell sensitization was as previously described.[2] In all experiments involving EAC'_1 care was taken to use only EA sensitized in the presence of Na_3 HEDTA.[3]

Preparation of sensitized red cell-complement component intermediates (EAC' . . .). The methods used to prepare $EA_{g.p.}C'_4$, $EA_{g.p.}C'_{1,4}$, $EA_{g.p.}C'_{1,4,2}$ and $EA_{hu}C'_1$ have been described in earlier publications.[3,4] $EA_{g.p.}C'_1$ were prepared either by use of guinea pig R4[9] or of purified guinea pig C'1.[4,10]

Hemolysis of EA was performed at 37° ± 0.1 for 60 minutes; EAC' . . . were subjected to hemolysis at 32° for the same period. The extent of hemolysis was measured by determining the optical density of the supernatant solution at 540 mμ. A Zeiss PMQII spectrophotometer and cuvettes with a 1 cm lightpath were used for all O.D. readings.

EXPERIMENTAL AND RESULTS

I. The Kinetics of Whole C' Inactivation by Poly I

Fifteen ml of a 1/20 dilution of guinea pig complement were incubated at varying temperatures with 1 ml of poly I solution containing 4 μmol P per ml. Timed serial 1 ml aliquots from this incubation mixture were added to 1 ml poly A solution (0.4 μmol P per ml) on ice. Serial 1 ml two-fold dilutions in BBS were then made, and to each dilution tube were added 5.5 ml BBS and 1.0 ml EA (5×10^8 per ml). The tubes were incubated at 37° for 1 hour and the number of residual $C'H_{50}$ were calculated for each time period. Similar aliquots of guinea pig serum were treated with poly (I + A) (4 μmol:6 μmol), and with poly A alone. A saline control experiment was also performed. The whole experiment was repeated using poly C in the place of poly A.

Serum treated with poly (I + A) showed no decrease in C' activity; nor did serum treated with poly A only. The loss of C' activity by poly I-treated C', measured in the presence of excess poly A as a "stopping" reagent, proceeded somewhat faster at 15°C than at 0°C (Figure 1). Increasing the temperature to 25° or 37° did not significantly increase the rate of C' inactivation. Similar results were obtained with poly C. Figure 2 shows the results of treating aliquots of 1/20 dilution of guinea pig serum with varying amounts of poly I at 15°C. The control concen-

*Kindly supplied by Dr. Irwin H. Lepow, Western Reserve University.

†Markham Laboratories, Chicago, Illinois.

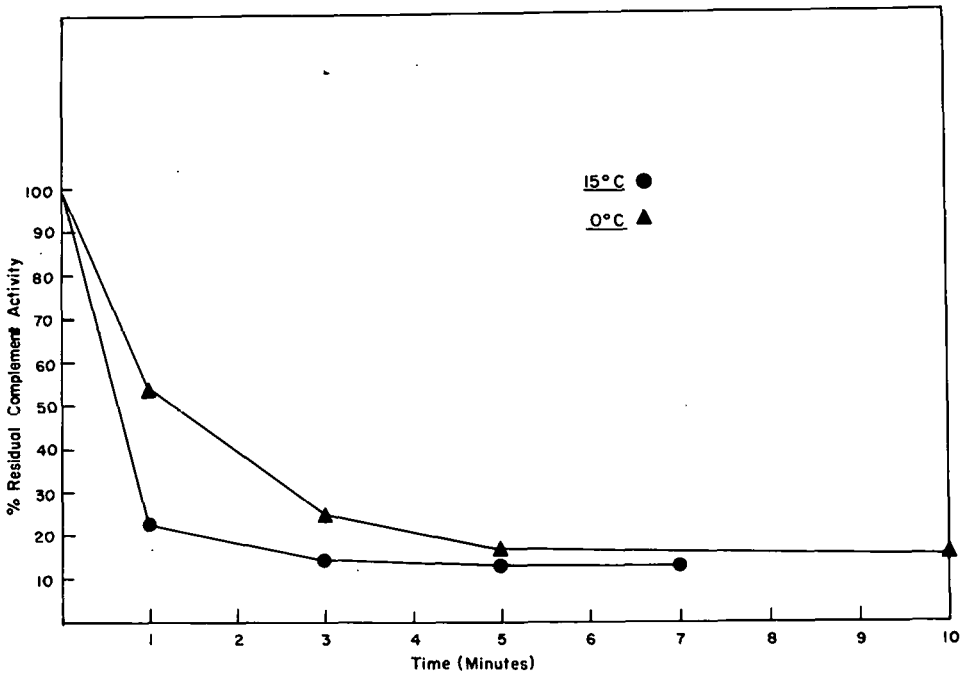

Figure 1. The effect of temperature on the rate of inactivation of whole serum C' by poly I. Poly A was used as a "stopping" agent. The system contained 12.2 $C'H_{50}$ ml at 0 time.

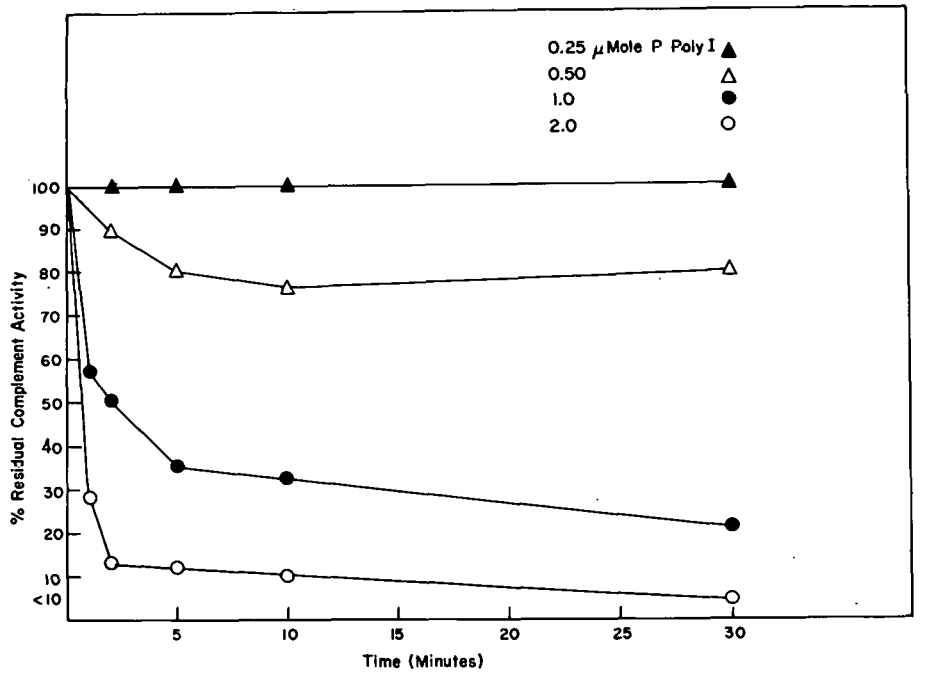

Figure 2. The effect of varying concentrations of poly I on the rate and extent of inactivation of whole serum C' at 15°C. Poly A was used as a "stopping" agent. The system contained 14.35 $C'H_{50}$ per ml at 0 time.

tration of C' was 14.35 $C'H_{50}$; Following exposure to 0.0625 μmol P poly I per ml (total 1 μmol) for 30 minutes only 3.1 $C'H_{50}$ remained. Thus, in this system, 0.0056 μmol P poly I ($\frac{0.0625}{11.25}$) served to inactivate 1 $C'H_{50}$, a figure which is in reasonably close agreement to the value 0.0075 μmol P poly I per $C'H_{50}$ obtained by another procedure.[2]

These results indicated that poly A could serve as a "stopping" reagent to prevent further action of poly I as an Anti-C' agent. Furthermore, the addition of poly A to a poly I-inactivated C' system would not reverse the Anti-C' effects of poly I. Since the return of C'1 or C'1q activity following inactivation by poly I and neutralization by poly A may require up to 24 hours,[1] an aliquot of diluted guinea pig serum was treated with poly I, followed by excess poly A addition, and the residual C' activity was measured immediately after the addition of poly A, and at 24 hours later. The results, shown in Table 1, reveal a decline in residual C' activity with the passage of time, rather than the reversal phenomenon noted with purified C'1 or C'1q.

Table 1

LYSIS OF EA IN POLY I-TREATED C' IMMEDIATELY AND 24 HOURS AFTER THE ADDITION OF EXCESS POLY A AS A "STOPPING" AGENT

Dilution of starting material	Time after the addition of poly A			
	5 minutes EA lysis*		24 hours EA lysis*	
	poly I C'	Control C'	poly I C'	Control C'
undil.	0.780	1.295	0.082	1.257
1:2	0.356	1.257	0.014	1.272
1:4	0.091	1.234	0.000	1.260
1:8	0.010	0.860	0.000	0.796
1:16	0.002	0.118	0.000	0.099

*Optical density 540 mμ. 100% lysis yields O.D. 1.270.

30 ml 1:100 guinea pig serum was exposed to 2 μmol P poly I for 20 minutes at 25° C. 3 μmol P poly A were added, and after 5 minutes at 25°, the mixture was placed at 1° and kept for 24 hours. A control C' aliquot was treated in similar fashion without poly I addition. The lytic test was performed by adding 2.5 ml of each dilution to 5 x 10^8 EA in a final volume of 4.0 ml.

II. Residual C' Component Activity in Poly I-inactivated Serum

Attention was next directed to identification of the C' component(s) that were absent from whole serum C' which had first been exposed to poly I, and then to a molar excess of poly A. EAC' . . . were employed and their lysis in a poly I-poly A treated serum aliquot was compared with their lysis in a control C' aliquot, and with the lysis of EA in the poly I-treated C'. The experimental protocol is presented in flow diagram form for greater clarity:

Experimental (poly I + poly A)

30 ml g.p. serum (1/20 dilution in BBS) + 2.5 ml poly I (4 μmol P per ml)

Incubate 30 minutes at 15°. Place on ice.

Add 3.0 ml poly A (4 μmol P per ml)

Remove three 10-ml aliquots.

10 ml	10 ml	10 ml
Add 0.5 ml BBS + 50 ml BBS	Add 0.5 ml 0.15 M Na_3 HEDTA + 50 ml Na_3 HEDTA BBS	Add 0.5 ml 0.15 M Na_2Mg EDTA + 50 ml Na_2Mg EDTA BBS

Make 30 ml serial two-fold dilutions, in appropriate buffered saline, of each of the above 3 solutions (1:1 → 1:32)

Add 5 ml of appropriate dilution to 1 ml 5 x 10^8 EA or EAC' . . . .

Incubate 60 minutes at 32°.

Control

30 ml g.p. serum (1/20 dilution in BBS) + 2.5 ml BBS

Incubate 30 minutes at 15°.

Place on ice.

Add 3.0 ml poly A (4 μmol P per ml). Remove three 10-ml aliquots and treat the same as the experimental group.

The results are shown in Figure 3 (A-D). The lysis of $EA_{g.p.}C'_{1,4}$ and $EA_{g.p.}C'_{1,4,2}$ in the BBS C' dilution are not depicted because they add nothing to the conclusions that can be drawn from the behavior of these cells in the appropriate EDTA buffer. Since the poly I-treated serum lost none of its ability to lyse $Ea_{g.p.}C'_{1,4,2}$ (in Na_3 EDTA BBS) or $EA_{g.p.}C'_{1,4}$ (in Na_2Mg EDTA BBS) we may conclude that poly I treatment of C' does not inactivate C'2 or C'3. EA lysis in BBS is, as expected, largely abolished by exposure to poly I. Of greater interest is the behavior of $EA_{g.p.}C'_1$ and $EA_{g.p.}C'_4$ in the BBS dilutions. Persensitizing the cell with C'1 partially restores lysis in BBS dilutions. This is not surprising since the ability of poly I to inactivate C'1 has already been amply demonstrated.[11,1] Persensitizing the cell with C'4, however, results in an even greater restoration of lysis, suggesting that poly I treatment of whole C', in addition to partially (or reversibly) inactivating C'1, also destroys C'4 activity. Further support for this conclusion is found when the behavior of $EA_{g.p.}C'_1$ is examined in the Na_2Mg EDTA BBS diluted C' reagents. Here, accepting that C'2 and C'3 activity is undiminished in the poly I-treated C', and recognizing that the presence of EDTA excludes any residual C'1 from functioning, the failure of $EA_{g.p.}C'_1$ to lyse in the poly I-treated C' can only mean that C'4 activity in the latter is diminished. In the experiment described below the advantage of EAC'_4 over EAC'_1 in restoring lysis to a poly I-inactivated C' reagent is even more pronounced.

III. The Effect of EDTA on C' Inactivation by Poly I

The polynucleotide was mixed with C' both in BBS, and in Na_3 HEDTA BBS, and following incubation, a molar excess of poly A was added. The samples (and appropriate controls) were then extensively dialyzed to remove EDTA and replenish Ca^{++} and Mg^{++}. Following this, their capacity to hemolyze EA, $EA_{hu}C'_1$ and $EA_{g.p.}C'_4$ was tested. The protocol of this experiment follows:

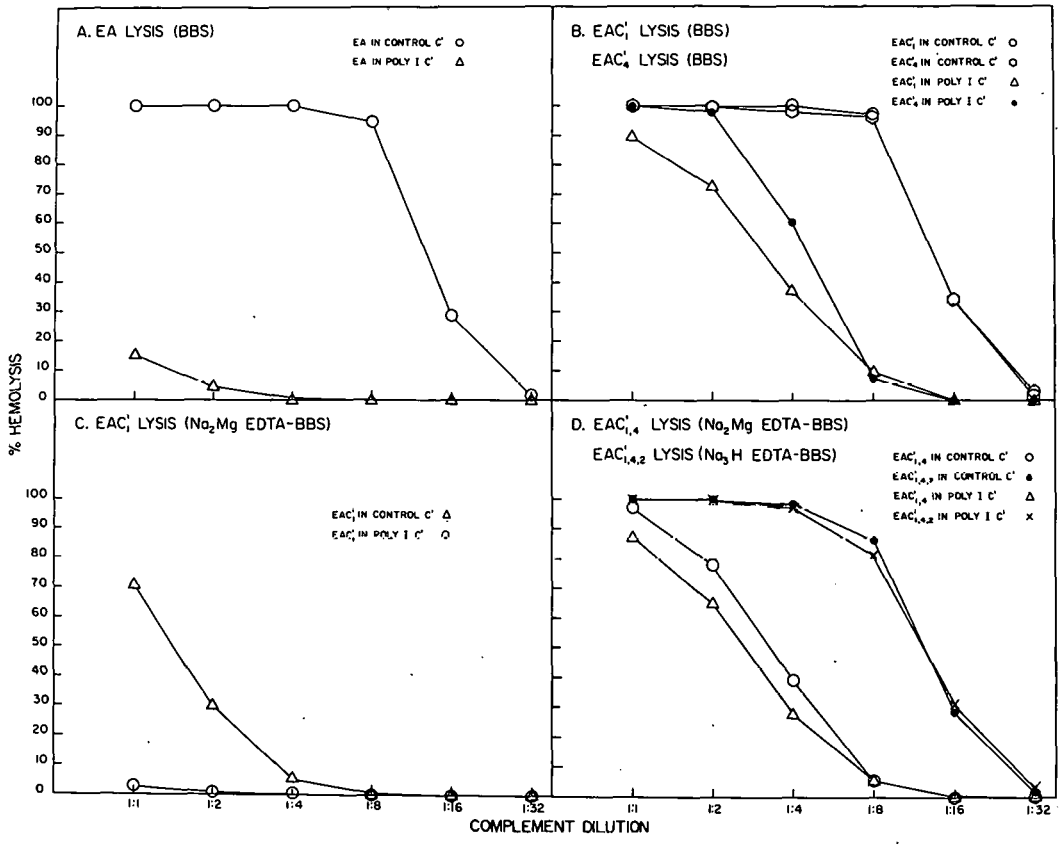

Figure 3. The ability of poly I-inactivated serum (to which poly A had been added as a "stopping" agent) to support the hemolysis of EA and various EAC' . . . intermediates. Exposure of serum to poly I was carried out in the presence of Ca^{++} and Mg^{++}.

Two 30 ml 1:100 guinea pig serum aliquots (Na_3 HEDTA BBS)		Two 30 ml 1:100 guinea pig serum aliquots (BBS)	
+	+	+	+
0.5 ml poly I (4 μmol P per ml)	0.5 ml Na_3 HEDTA BBS	0.5 ml poly I (4 μmol P per ml) Incubate 30 minutes at 15°C. Add:	0.5 ml BBS
0.75 ml poly A (4 μmol P per ml)	0.75 ml Na_3 HEDTA BBS	0.75 ml poly A (4 μmol P per ml)	0.75 ml BBS

Dialyze for 24 hours at 4°C vs 2 4-liter changes of BBS containing 6.5×10^{-4} M Ca^{++} and 5×10^{-4} M Mg^{++}. Divide each into two portions. Make serial dilutions of one in BBS. Add 0.15 M Na_2Mg EDTA to the other (final concentration 2.6×10^{-2} M) and make serial dilutions in Na_2Mg EDTA BBS (2.6×10^{-2} M). Test EA, $EA_{hu}C'_1$ and $EA_{g.p.}C'_4$ with appropriate reagents (1.5 ml cells (5×10^8) + 2.5 ml reagent dilution). Incubate 1 hour at 32°.

The results are shown in Figure 4 (A-D). The effects of poly I treatment of C' in the presence of Ca^{++} and Mg^{++} are similar to those described in the preceding section, although the amount of lysis restored (in BBS) by $EA_{hu}C'_1$ is less, and the capacity to restore lysis by EAC'_4

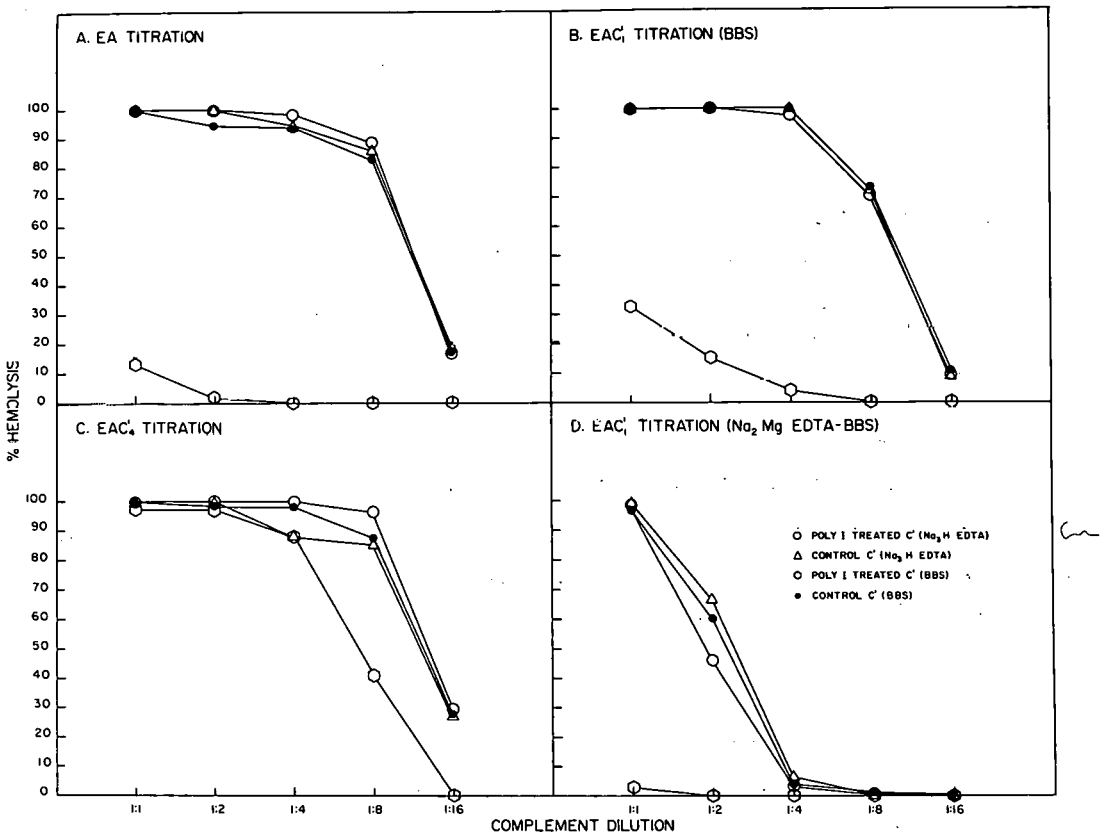

Figure 4. The effect of Na_3HEDTA on the inactivation of serum C' by poly I. Following the addition of poly A as a "stopping" agent, the Na_3HEDTA was removed by dialysis and the appropriate titrations were performed.

in BBS is substantially greater. These findings are interpreted as indicating that here there are greater C'4 inactivation, and more complete reversal of C'1 inactivation than in the preceding experiment. It is of great interest that poly I treatment of C' in the presence of Na_3HEDTA prevents the inactivation of C' activity thus implying that such inactivation is dependent upon divalent cations. Since the same result was obtained (lack of C' inactivation) when Na_2Mg EDTA was used in place of Na_3 HEDTA during exposure of C' to "active" poly I, the dependence of this inactivation phenomenon on Ca^{++} was established. In all experiments involving exposure of C' to active poly I in the presence of EDTA it was, of course, necessary to add the poly A prior to dialysis; if poly A was added after dialysis, or if no poly A was added, C' inactivation occurred.

IV. Studies on the Mechanism of C'4 Inactivation by Poly I

From the data thus far described, and from data on the effects of poly I on C'1,[1] an analogy emerged between the effects of poly I on C' and those of γ-globulin aggregates or of antigen-antibody aggregates. The hypothesis was that poly I inactivation of C'4 was mediated via its interaction with C'1 and the conversion of C'1 by poly I to an "active" esterase form, the latter then being responsible for C'4 inactivation. Poly I had previously been shown to be non-inhibitory to C'1 esterase activity as measured by synthetic amino acid ester hydrolysis.[11] The following experiment was designed to see whether poly I could diminish the capacity of purified C'1 esterase to inactivate C'4.[12]

C'1 esterase (8 units per ml) is diluted in two-fold serial fashion (1:1 → 1:256).

Add 0.1 ml of each C'1 esterase dilution to 0.1 ml BBS.

Incubate 15 minutes at 25°.

Add 0.1 ml of each C'1 esterase dilution to 0.5 ml poly I (4 μmol P per ml).

Add 0.05 ml of poly A (6 μmol per ml).

Incubate 5 minutes at 25°.

Add 0.4 ml human serum (heated 56°, 30 minutes) to each tube as a source of C'4.

Incubate 30 minutes at 0°C.

Dilute all tubes 1:8 with BBS.

To 0.2 ml of each 1:8 dilution add 0.1 ml human R4, 0.2 ml BBS and 1.0 ml EA (2.5×10^8).

Incubate 30 minutes at 37° and estimate the degree of lysis by comparison with visual standards.

The results of this experiment indicate that poly I does not inhibit the capacity of purified C'1 esterase to inactivate C'4 (Table 2).

Table 2

THE EFFECT OF POLY I ON THE ABILITY OF C'1 ESTERASE TO INACTIVATE C'4

Dilution of C'1 esterase	% lysis*	
	Control C'1 esterase	poly I-treated C'1 esterase
undil.	0	0
1:2	0	0
1:4	0	0
1:8	0	0
1:16	0	0
1:32	10	10
1:64	30	40
1:128	50	50
1:256	> 50	> 50

*By visual comparison with standards.

Poly I cannot prevent the inactivation of C'4 by purified C'1 esterase. See text for details of the experimental protocol.

If the inactivation of C'4 by poly I is mediated via C'1 "activation," then poly I should be incapable of inactivating purified C'4 in the absence of C'1. The experiment shown in Table 3 was performed to test this hypothesis: R4 was used as a source of C'1 during the phase of C'4 exposure to poly I (purified C'1 was not used since it alone is capable of C'4 inactivation). The exposure of purified C'4 to poly I in the absence of C'1 (R4) did not significantly reduce its ac-

Table 3

THE INACTIVATION OF PURIFIED C'4 BY POLY I IN THE PRESENCE OF A SOURCE OF C'1

	Tube #							
	1	2	3	4	5	6	7	8
Reagent, ml								
R4 (1:10)	1.0	-	1.0	-	1.0	-	1.0	-
C'4 (1:10)	-	1.0	1.0	-	-	1.0	1.0	-
Buffered saline	1.0	1.0	-	2.0	1.1	1.1	0.1	2.1
Poly I (4 μmol P per ml)	0.1	0.1	0.1	1.1	-	-	-	-
Incubate 15° C, 30 minutes. Add								
Poly A (4 μmol P per ml)	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Incubate 15° C, 10 minutes Add 1.0 ml of each of above mixtures (1-8) to separate tubes containing 1.0 ml EA (5×10^8) + 2 ml R4 (1:50). Incubate 37°, 1 hour.								
O.D. 540 mμ	0.010	0.729	0.075	0.000	0.005	0.782	1.237	0.002

The exposure of purified C'4 to poly I does not reduce C'4 activity (compare tubes 2 and 6). The exposure of C'4 to poly I in the presence of a source of C'1 results in almost complete destruction of C'4 activity (compare tubes 3 and 7). Both the C'4 and R4 were of guinea pig origin.

tivity (compare tubes 2 and 6); if purified C'4 was exposed to poly I in the presence of a source of C'1, C'4 activity was almost entirely destroyed (compare tubes 3 and 7).

DISCUSSION

The data presented demonstrate that in addition to inactivation of C'1,[11,1] poly I causes the inactivation of C'4 when allowed to react with whole C'. On the basis of the present evidence the following brief description of the effects of poly I on the C' system may be offered: Free poly I is capable of inactivating whole C' activity; approximately 0.0075 μmol P poly I will inactivate 1 $C'H_{50}$ of human or guinea pig C'.[2] The inactivation of C' activity in whole serum is a time-consuming process which can be slowed slightly by low temperatures. Poly I, by virtue of its effects on EAC' . . ., is a selective inhibitor of C'1 hemolytic activity.[11] The inhibition of C'1, when studied by means of the purified component, is exceedingly potent. The inhibition of C'1 hemolytic activity by poly I results from the polymer's selective interaction with C'1q. To a variable extent and under appropriate conditions (e.g., subsequent addition of poly A) the inactivation of C'1q and of hemolytic C'1 activity are reversible phenomena.[1] Poly I does not inhibit C'1 esterase activity, as measured either by hydrolysis of a synthetic amino acid ester substrate,[11] or by the ability of C'1 esterase to inactivate C'4. It has no effect on C'1 esterase serum inhibitor.[11] In whole serum, poly I not only reversibly inactivates C'1 but also causes the irreversible destruction of C'4. When exposed to C'4, either as EAC'_4[11] or as purified C'4 in solution, poly I does not affect this C' constituent; the inactivation of C'4 appears to be depend-

ent upon a source of C'1. The inhibition of C' activity in a native C' system can be entirely prevented by EDTA; the critical divalent cation is Ca^{++}.

The pattern is familiar and resembles in many respects the inactivation (or fixation) of hemolytic C' activity by aggregated γ-globulin[13-15] and antigen-antibody aggregates.[5,16-18] Thus the interaction of poly I with the C'1q portion of the C'1 molecule in the presence of Ca^{++} leads, in some poorly understood way, to the "activation" of the C'1 molecule, rendering it hemolytically inactive in a whole C' system. C'1q (11S component) appears to function as, or bear the binding site for,[4] the reaction of C' with properly oriented or altered γ-globulin molecules.[19-21] At the same time this poly I-C'1 interaction activates C'1 proesterase, and the active C'1 esterase in turn proceeds to inactivate C'4.[22] Poly I differs in certain respects from the action of γ-globulin aggregates or antigen-antibody aggregates. Most important of these is the reversibility of its binding to C'1q by poly A or poly C.[1] In addition, we have as yet detected no changes of C'2 activity in poly I exposed C'. Of interest in this regard is Turk's report that two conventional antigen-antibody systems are capable of fixing C'1 and C'4, but can fix little, if any, C'2.[23] The possibility that the effects of poly I are in some way mediated via 7S γ-globulin has been explored and seems remote.[11]

Poly I appears to be a substance, apart from properly altered or oriented γ-globulin, capable of initiating many of the early events involved in the participation of C' in immune systems. This capacity, which is also demonstrable in vivo,[11] appears to be due to its ability to interact specifically with C'1q. The demonstration that other substances than γ-globulin exist which are capable of initiating activation of the complement system, raises the possibility that such substances, provided they could enter the blood stream, might cause pathological events, such as anaphylactoid reactions,[24] or the deposition of C' proteins in tissues[25,26] without, in the strictest sense, being related to immune phenomena.

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BIOLOGIC PROPERTIES OF POLYNUCLEOTIDES. VII. VASCULAR PERMEABILITY IN GUINEA PIG SKIN INDUCED BY POLYINOSINIC ACID*

By

S. Yachnin† and J. M. Ruthenberg

On the basis of previous studies on the anticomplementary effects of various synthetic polynucleotides[1-5] an analogy was drawn between the effects of poly I on the C' system and those of antigen-antibody aggregates or heat aggregated gamma globulin. It was postulated that the interaction of poly I with the C'1q portion of the C'1 macromolecule blocks the usual site of attachment of C'1 to sensitized sheep erythrocytes. At the same time, and in a presently little understood manner, this poly I - C'q interaction leads to activation of the C'1 molecule (C'1a), and conversion of C'1 proesterase to an active esterase form. C'1 esterase in turn irreversibly inactivates C'4.[5]

While the participation of complement in the pathogenesis of vascular permeability reactions has been suggested by Osler et al. on the basis of their studies on passive cutaneous anaphylaxis in the rat,[6] later work has indicated that in the guinea pig alternative mechanisms may be involved.[7] Nevertheless, Ratnoff and Lepow have recently demonstrated that the intracutaneous injection of a highly purified preparation of human C'1 esterase is capable of evoking vascular permeability in the guinea pig.[8] The present study describes the enhancement of vascular permeability in guinea pigs by intracutaneous injection of poly I, thus supporting the hypothesis that poly I serves as an activator of C'1.

MATERIALS AND METHODS

The methods of synthesis or isolation of the various polynucleotides under study together with a summary of some of their physical properties have been published.[1,2] The polynucleotides were dissolved in 0.15 M NaCl at a maximum concentration of 10 μmole P/ml. Partially purified human C'1 was prepared by the method of Lepow, Ratnoff, Rosen and Pillemer[9] and was activated to the esterase form as described by the same authors. Two separate preparations contained 24 and 15 units of C'1 esterase/ml.[10] Purified C'1 esterase serum inhibitor‡ was prepared by the method of Pensky, Levy, and Lepow.[10,11] Aggregated gamma globulin was prepared as outlined by Müller-Eberhard and Kunkel[12] utilizing heat aggregation and Na_2SO_4 fractionation. The starting material, human 7 S gamma globulin (Cohn Fraction II) was obtained from Pentex Laboratories. Histamine acid phosphate (Eli Lilly and Company) and triprolidine HCl§ were employed in certain experiments.

Vascular permeability was tested by the technique of Miles and Wilhelm.[13] Male albino

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†John and Mary R. Markle Scholar in Academic Medicine.

‡Kindly supplied by Drs. C. F. Hinz, Jr., and J. Pensky, Western Reserve University.

§Kindly donated by Burroughs Wellcome and Company, Tuckahoe, N.Y. as "Actidil."

guinea pigs weighing between 400 and 800 g were used. All measurements of lesions were made 30 min after injection. In all other respects the techniques were similar to those described by Ratnoff and Lepow.[8]

RESULTS

1. The effects of various polynucleotides on vascular permeability. The effects of increments of intracutaneously injected poly I on vascular permeability are shown in Figure 1. The lesion evoked by one-tenth μmole poly I was not significantly greater than that evoked by the control saline injection, but the intensity of the blue area was usually greater. The injection of 1 μmole poly I consistently elicited maximum intensity reactions of 8.5 to 11 mm in diameter.

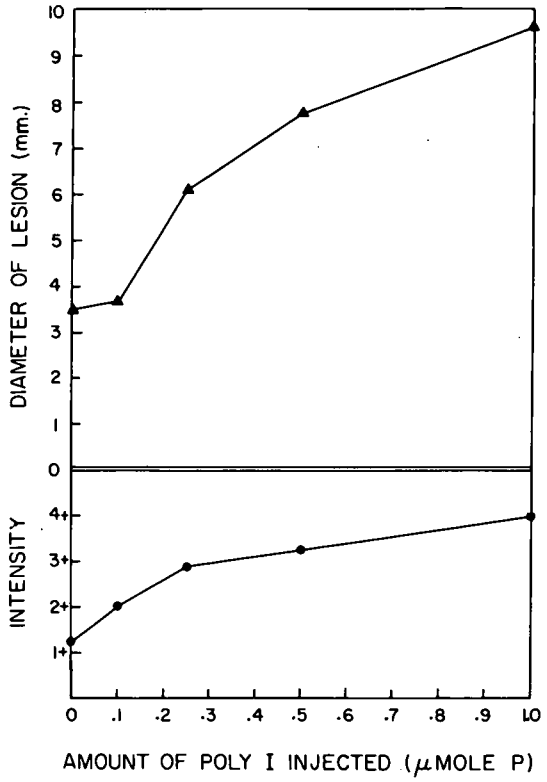

Figure 1. The ability of increments of intracutaneously injected poly I to promote vascular permeability.

The following polynucleotides, both naturally occurring and synthetic, were incapable of evoking significant vascular permeability in amounts up to 1 μmole P per injection site: polyadenylic acid (poly A); polycytidylic acid (poly C); polyuridylic acid (poly U); RNA derived from rat liver, guinea pig liver, and mouse spleen; and apurinic acid prepared from cell thymus DNA. A comparison of the effects of poly I and various other polynucleotides on vascular permeability is shown in Figure 2.

2. The effect of helix formation with poly A or poly C. The ability of poly I to interact with the C' system is abolished by incorporating poly I into a hydrogen-bonded helical structure with poly A or poly C.[1] From the data in Table 1, it can be seen that poly I incorporated into such

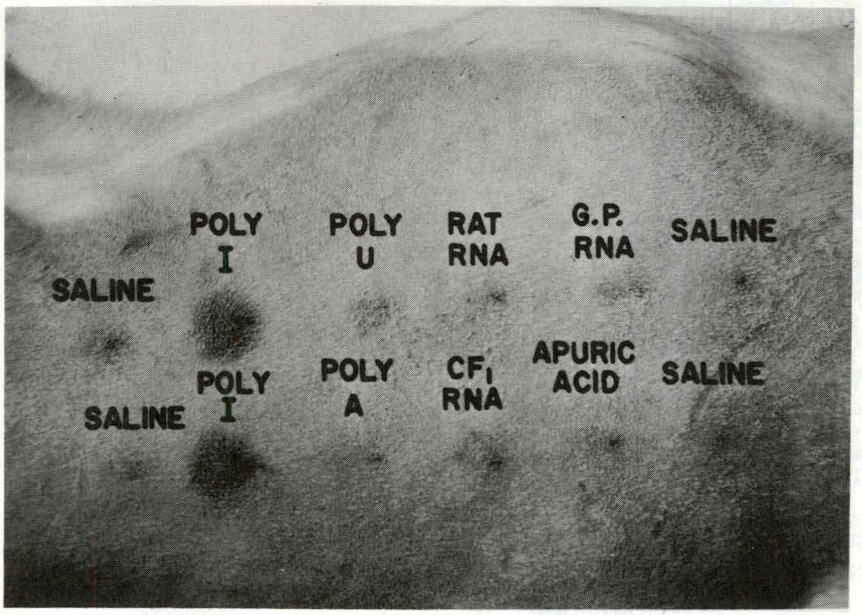

Figure 2. A comparison of the effects of various intracutaneously injected polynucleotides upon vascular permeability. One μ mole P of each polynucleotide was injected into the dorsal skin of a guinea pig. (CF_1 RNA - RNA from mouse spleen; apuric acid - apurinic acid derived from calf thymus DNA.)

Table 1

THE EFFECT OF HELIX FORMATION WITH POLY A OR POLY C UPON THE ABILITY OF POLY I TO EVOKE VASCULAR PERMEABILITY

Material injected	Amount injected μmole P	Diameter of lesion mm	Intensity of lesion (1-4)
Saline control		3.48	1+
poly I	0.5	8.75	4+
poly (C + I)	1.0*	3.25	1.5+
poly (A + I)	1.0*	3.5	1+
poly A	0.5	3.0	1+
poly C	0.5	2.5	1±

*0.5 μmole P each polynucleotide in a 1:1 preformed helix.

helical structures was unable to evoke vascular permeability.

3. The effect of delay between injection of poly I and intravenous administration of dye on the spreading reaction. The ability of C'1 esterase to cause spreading of intravenously administered dye diminishes as the period between C'1 esterase injection and dye administration increases.[8] A similar phenomenon was noted with poly I although the rate of decrease in lesion size was slower than that described by Ratnoff and Lepow for purified C'1 esterase (Figure 3). In addition, inspection of the site of poly I injection prior to dye administration in the 22-minute and later dye injections revealed obvious evidence of local edema and mild erythema.

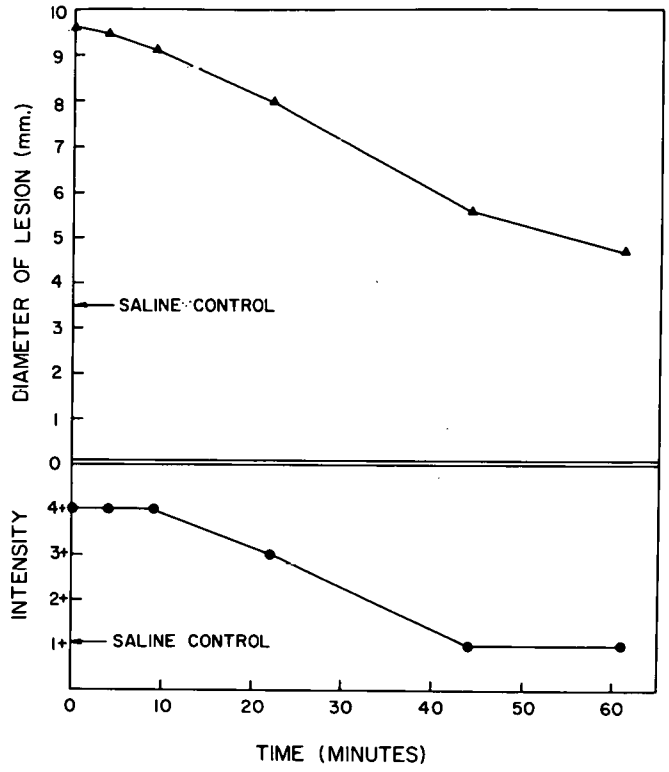

Figure 3. The effect of delay between injection of poly I and the intravenous administration of dye on the spreading reaction.

4. The effect of antihistamine administration. The vascular permeability evoked by purified C'1 esterase is abolished by pretreating the test animals with as little as 0.1 mg/kg triprolidine HCl.[8] Table 2 shows that as much as 10 mg/kg of the same antihistamine failed to inhibit the vascular permeability evoked by poly I.

The inability of antihistamine to inhibit the vascular permeability evoked by poly I emerged as a major difference between the latter material and purified C'1 esterase. Experiments were therefore performed testing the ability of poly I, heat aggregated human 7 S gamma globulin, purified activated human C'1 (C'1a), and histamine diphosphate to evoke vascular permeability with and without antihistamine pretreatment. All the test materials were studied simultaneously in any single test animal, with the results shown in Table 3. Pretreatment with 1 mg/kg triprolidine was effective in abolishing only that vascular permeability elicited by histamine diphosphate injection. The effects of purified C'1a were only slightly inhibited by antihistamine pretreatment, in striking contrast to the observation of Ratnoff and Lepow[8] who utilized highly purified C'1 esterase rather than the entire C'1 macromolecule. The ability of aggregated 7 S gamma globulin to provoke vascular permeability was also only partially inhibited. Poly I was unaffected.

5. The effect of C'1 esterase serum inhibitor. Equal volumes of test material were mixed with a solution of C'1 esterase serum inhibitor, incubated at room temperature for 15 min, and tested for their capacity to evoke vascular permeability. The results are shown in Table 4. The ability of C'1a to evoke vascular permeability is largely inhibited, while poly I and aggregated gamma globulin are unaffected.

Table 2

THE EFFECT OF AN ANTIHISTAMINE AGENT, TRIPROLIDINE HCl, UPON THE ABILITY OF POLY I TO EVOKE VASCULAR PERMEABILITY

Amount of poly I injected μmole P	Pretreatment	Diameter of lesion mm	Intensity of lesion (1-4+)
none (saline control)	none	3.48	1+
1	none	9.61	4+
1	triprolidine 0.1 mg/kg	9.69	4+
1	triprolidine 0.25 mg/kg	9.88	4+
1	triprolidine 1 mg/kg	10.35	4+
1	triprolidine 10 mg/kg	9.43	4+

Table 3

THE EFFECT OF PRETREATMENT WITH AN ANTIHISTAMINIC AGENT UPON THE CAPACITY OF VARIOUS MATERIALS TO EVOKE VASCULAR PERMEABILITY*

Material injected	Amount injected	Pretreatment			
		None		Triprolidine HCl 1 mg/kg	
		Diameter of lesion mm	Intensity of lesion (1-4+)	Diameter of lesion mm	Intensity of lesion (1-4+)
Experiment A					
Saline	0.1 ml	2.4	1+	2.4	1+
Poly I	1 μmole	8.75	4+	8.42	4+
C'1a	2.4 U	9.33	4+	7.67	4+
Aggregated γ globulin	33 μg	9.33	2+	6.33	1.6+
Histamine acid phosphate	2.75 μg	8.87	3+	3.75	1.6+
Experiment B					
Saline	0.1 ml	2.4	1+	2.4	1+
Poly I	1 μmole	7.58	3.8+	9.08	4+
C'1a	1.5 U	7.83	3.7+	7.25	4+
Aggregated γ globulin	45 μg	8.43	3+	5.25	1+
Histamine acid phosphate	2.75 μg	7.58	2.7+	3.58	1+

*Two separate experiments are presented, each using a different preparation of C'1a. Each result represents the average of 6 injections in 3 animals.

Table 4

THE EFFECT OF C'1 ESTERASE SERUM INHIBITOR UPON THE ABILITY OF VARIOUS MATERIALS TO EVOKE VASCULAR PERMEABILITY*

Material	Amount injected		Diameter of lesion mm	Intensity of lesion (1-4+)
	Alone	With C'1† esterase inhibitor		
Saline	0.1 ml		3.17	1.6+
Saline		0.1 ml	3.0	1.6+
Poly I	0.5 μmole		8.0	4+
Poly I		0.5 μmole	7.67	4+
Aggregated γ globulin	25 μg		8.0	2+
Aggregated γ globulin		25 μg	8.3	2+
C'1a	0.75 U		8.3	2.3+
C'1a		0.75 U	4.17	2+

*Each result represents the average of one injection in 3 test animals.

†1.1 U C'1 esterase serum inhibitor.

DISCUSSION

That poly I alone of the polynucleotides examined, is capable of eliciting vascular permeability reactions, while it is at the same time the only material tested capable of interacting with the complement system, suggests a relationship between the two phenomena. This possibility is strengthened by the observation that the maneuver which abolishes the ability of poly I to interact with whole C'[1] and purified C'1 or C'1q,[4] complexing with poly A or poly C, also destroys its capacity to evoke vascular permeability. In the light of earlier observations that C'1s (C'1 proesterase[14]) is incapable of evoking vascular permeability unless it is first converted to an active esterase form,[8] the ability of poly I to produce vascular permeability provides further, albeit still indirect, evidence that poly I serves as an activator of C'1. These observations extend the analogy drawn elsewhere between the interaction of poly I with the complement system and that of antigen-antibody aggregates or heat activated gamma globulin,[5] since the latter also serve as activators of C'1 and are capable of evoking vascular permeability which is presumably mediated via the C' system.[15-20]

The fact that the size and intensity of the blue area evoked by poly I diminish with the increase of time between its intracutaneous administration and the intravenous administration of dye does not necessarily mean that its effect is evanescent, since obvious local reaction had appeared prior to dye injection. Failure of dye to spread in the preformed lesion may simply represent impedance to further exudation of vascular fluid into a site of intracutaneous engorgement produced by prior leakage of non-dye containing fluid. The so-called "evanescent" effects of intracutaneously administered histamine and purified C'1 esterase[8] may represent a similar mechanical interference with the exudation of late-administered dye, and not a true lack of vascular permeability effects.

Poly I, antigen-antibody aggregates, and heat aggregated gamma globulin share potent com-

plement fixing properties, together with the ability to interact with C'1. In addition, all three are capable of inducing vascular permeability when injected intracutaneously. This latter property is potentially mediated through the complement system. Since activation of C'1 is the early common pathway for initiation of complement action, the ultimate mechanism involved in eliciting vascular permeability by poly I and gamma globulin aggregates may be the same as that involved in the vascular permeability evoked by purified C'1 esterase. Nevertheless, the fact that antihistamines abolish the capacity of the latter to produce vascular permeability,[8] without affecting poly I in a similar fashion, suggests that focal intracutaneous C'1 activation may produce its effects through mechanisms other than histamine release. While the purified C'1a used in these experiments cannot be considered a pure substance, and may in fact be contaminated by other plasma factors such as Hageman factor,[9] kallikrein and PF/Dil, the latter two of which are not inhibited by antihistamine,[21] it is nevertheless possible that the inability of antihistamines to inhibit completely the vascular permeability effects of 2.4 or 1.5 C'1a esterase units may reflect a difference in the mechanism by which whole C'1a (which is made up of C'1q, C'1r, and activated C'1s[13] acts, as contrasted to purified C'1 esterase (presumably equivalent only to activated C'1s).[22] It is possible that C'1 esterase, which by itself apparently lacks the ability to liberate histamine,[8] may serve to bring about the activation of a later complement component (?C'3)[6] which in turn liberates the actual permeability factor involved in endothelial cell injury, and which could conceivably involve more than histamine (?Bradykinin). The discrepancy in behavior between purified C'1 esterase and whole C'1a in regard to inhibition by antihistamine might reflect subtle differences in the manner of their interaction with later steps in the evolution of total complement activity, related in this particular experimental situation to differences in the molecular weight of the active esterase moiety,[23] or the presence or absence of skin binding sites. Since C'1 esterase is the sole plasma permeability factor presently obtainable in sufficiently pure form, and since indirect methods using C'1 esterase inhibitor or DFP, which inhibit C'1a, PF/Dil and kallikrein[8,21,24] lack sufficient specificity, the differences in the mode of action of C'1a and purified C'1 esterase in inducing vascular permeability are the proper subject for further investigation. In addition, considering the many common features shared by the two materials[25,26] it would seem that the relationship between kallikrein and C'1a - C'1 esterase would warrant further careful study.

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ENZYMATIC SYNTHESIS OF RIBONUCLEIC ACID. IV. THE DEOXYRIBONUCLEIC ACID-DIRECTED SYNTHESIS OF COMPLEMENTARY CYTOPLASMIC RIBONUCLEIC ACID COMPONENTS*

By

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The ribonucleic acid product formed by ribonucleic acid polymerase resembles its deoxyribonucleic acid template in base composition and in base sequence.[1-6] The reaction in vitro results in the synthesis of RNA chains which are complementary to both DNA strands.[6] Evidence for this originates from the formation of highly ordered RNA structures when the RNA polymerase product is annealed with itself,[7] and from base composition analysis of the polymerase product when primed by single or double stranded ΦX174 DNA.[8,9] Self-annealed synthetic complementary ribonucleic acid exhibits properties very similar to helical DNA.[7] Although RNA polymerase catalyzes the synthesis of two complementary ribopolymers in vitro, studies with bacteriophage systems have led to the conclusion that informational RNA results from the transcription of only one DNA strand in vivo.[10-12] It is not yet certain whether the bacteriophage systems represent a general transcription mechanism or a specialized case. Recently, Hayashi, Hayashi, and Spiegelman[13] reported that when a "replicating form" of ΦX174 DNA is used in the RNA polymerase reaction in vitro, only one DNA strand is copied.

Observations by other investigators have led to the belief that the synthesis of most, if not all, cytoplasmic RNA is under the direction of DNA. Enucleated cells show a rapid drop in the content of their cellular RNA.[14] Actinomycin D, an inhibitor of RNA polymerase, prevents the synthesis of cytoplasmic RNA when given to whole cells.[15] Other evidence in support of this concept is the demonstration of specific RNA-DNA hybrid formation between microbial cytoplasmic ribonucleic acids and homologous DNA.[16-18] Hybrid formation of this type implies the presence of nucleotide sequences in cytoplasmic RNA which are complementary to base sequences in DNA. Therefore, DNA directs not only the synthesis of template RNA for protein synthesis, but ribosomal and transfer ribonucleic acids as well.

The high degree of self-complementarity among the RNA chains formed by RNA polymerase suggested the possibility that the self-complementary ribonucleic acid product might contain one strand which was identical in base sequence with ribosomal and transfer RNA, and the other strand complementary to these cytoplasmic ribopolymers. A demonstration of synthetic ribonucleic acid complementarity to natural cytoplasmic ribonucleic acids would be evidence that the assembly of polyribonucleotides in vitro results in the synthesis of specific nucleic acid chains, and would strengthen conclusions concerning the origin of cytoplasmic RNA. In this paper, we shall describe experiments which suggest that part of the RNA synthesized on a DNA template, in vitro, is complementary to homologous 23 S and 16 S ribosomal RNA, and to homologous transfer RNA.

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EXPERIMENTAL PROCEDURE

Materials and methods. The RNA polymerase used in this report was purified from extracts of Micrococcus lysodeikticus by Method A described previously[19] with the following modifications. Prior to treatment with DEAE-cellulose, ammonium sulfate Fraction V was dissolved in 4 ml of 0.1 M Tris-HCl buffer, pH 7.5, and passed over a Sephadex C-75 column (3.5 x 80 cm) which had previously been equilibrated with 0.3 M ammonium sulfate neutralized with NH_4OH to pH 7.0. The enzyme was eluted at room temperature with this salt solution, and appeared after 180 ml had passed through the column. Ninety per cent of the activity was recovered in a volume of 70 ml, iced, and concentrated by the addition of solid ammonium sulfate to a final concentration of 40 per cent of saturation at 0°. The precipitate was collected by centrifugation and the Sephadex procedure repeated on a second column of equal size. The enzyme collected after the second Sephadex elution was stored in 50 per cent glycerol and treated with DEAE-cellulose as previously reported.[19] This procedure significantly reduced the ribonuclease activity associated with the enzyme preparation. On extensive incubation of the enzyme with ^{32}P-RNA, no loss in acid-insoluble counts was observed. However, some ribonuclease activity still remained as evidenced by a 50 per cent loss in plaque-forming units when 100 μg of enzyme were incubated for 20 minutes at 30° with 0.5 μg of infectious MSϕ2 RNA.* This loss in biological activity is equivalent to the action of 5×10^{-5} μg of pancreatic RNase as determined by assays under identical conditions.

Mammalian, bacterial, and viral DNA and RNA preparations were obtained as described elsewhere.†[20,21] Synthetic cRNA‡ was prepared with the purified RNA polymerase, and isolated in pure form by equilibrium centrifugation in CsCl as described previously.[6]

Growth of cells and RNA isolation. Escherichia coli B was grown on the synthetic medium C as described by Roberts, Abelson, Cowie, Bolton, and Britten.[22] ^{32}P-labeled ribosomal and transfer RNA were prepared in a manner similar to that reported by Yankofsky and Spiegelman.[16] Cultures of E. coli B in synthetic medium containing 0.02 M sodium phosphate were grown overnight to an optical density at 650 mμ of approximately 1. The cultures were then diluted in 15 ml of synthetic medium to an optical density of 0.02 and adjusted to contain 2×10^{-3} M inorganic phosphate with 20 mc of neutralized, carrier-free $^{32}P_i$. The cells were shaken at 37° for 5 to 6 hours until an optical density of 0.3 to 0.5 was obtained. Inorganic phosphate (1 mmole) was added and the cells were collected by centrifugation, washed once with synthetic medium, and resuspended in 30 ml of synthetic medium containing 0.10 M inorganic phosphate. The cells were grown for one generation, centrifuged, and stored overnight at -20°. This "chase" procedure was used to eliminate ^{32}P-labeled messenger RNA.[23-26]

The frozen pellet was thawed and suspended in 5 ml of a solution containing 0.02 M Tris (pH 7.5), 0.005 M $MgCl_2$, and 2.5 mg of lysozyme. A solution of 25 per cent Duponol was added to give a final concentration of 1 per cent, which resulted in a marked increase in viscosity, indicating cell lysis. The nucleic acid was isolated by four or five successive treatments with phenol, followed by three precipitations with ethanol.[27] The ^{32}P-nucleic acid was dried in a vacuum at

*MSϕ2 is an RNA-containing coliphage virus which requires the male strain of E. coli, either Hfr or F^+, as the host for propagation.

†E. coli DNA was supplied by Dr. E. P. Geiduschek, and turnip yellow mosaic virus and tobacco mosaic virus RNA were supplied by Dr. R. Haselkorn.

‡The abbreviation used is: cRNA, complementary ribonucleic acid.

room temperature for 30 minutes, and then dissolved in a small volume (less than 1 ml) of 0.01 M Tris, pH 7.5.

Transfer RNA. Transfer RNA was isolated from a portion of the ^{32}P-nucleic acid by chromatography on columns of methylated albumin as outlined by Sueoka and Cheng.[28] The labeled RNA fractions used had a single 4 S component on sucrose gradient centrifugation, and were shown to contain 2 to 3 per cent of ψ uridylic acid after alkaline hydrolysis and analysis by paper chromatography.[29]

Ribosomal RNA. Another portion of the phenol-prepared labeled nucleic acid was subjected to equilibrium centrifugation in CsCl, and isolated free of DNA as previously reported.[6] The isolated ^{32}P-RNA was then fractionated by sucrose gradient centrifugation into 23 S, 16 S, and 4 S components. The individual fractions of the 23 S and 16 S components were used in the annealing experiments reported here.

The concentration of each RNA fraction was determined optically at 260 mμ by the use of an extinction coefficient (for a 1-cm light path) of 25 per mg of RNA as determined by P_i analysis. Specific activity measurements were calculated from acid-precipitable radioactivity, and ranged from 2×10^5 to 8×10^5 c.p.m. per μg of RNA. The 23 S, 16 S, and 4 S labeled ribonucleic acids were resistant to DNase degradation but were completely susceptible to RNase and alkaline hydrolysis.

Annealing conditions. Mixtures of different natural or synthetic nucleic acids with the labeled cytoplasmic RNA components were made up to a final volume of 0.30 ml containing 0.2 M NaCl in 0.01 M Tris, pH 7.5. These mixtures were heated in a boiling water bath for 10 minutes, and then immediately placed in a 60-65° constant temperature bath and annealed overnight. In later experiments, slow cooling from 85° was used since it resulted in more extensive RNA-RNA complex formation. In some of the early experiments, the mixtures were quenched in ice after heating, just prior to annealing. However, this procedure was discontinued when it was found to have little effect on the amount of hybrid formed.

Assay for RNA-RNA hybrid formation. The annealing mixtures were cooled to room temperature and adjusted to 1.0 ml containing 5 μmoles of $MgCl_2$, 10 μg of pancreatic ribonuclease, and 1 μg of ribonuclease T_1. The vessels were incubated for 1 hour at 37° and then iced. Carrier yeast RNA (1 mg) was added and immediately followed by the addition of 0.10 ml of cold 50 per cent trichloroacetic acid. The acid-insoluble material was then prepared for counting as described elsewhere.[19] In the presence of pancreatic ribonuclease alone, the digestion of nonhybridized RNA was incomplete, leading to high base-lines which made detection of small quantities of RNA hybrids difficult. This was especially true in experiments with 4 S ^{32}P-RNA.

Ribonuclease T_1, prepared from Aspergillus oryzae, was purchased from the Sankyo Company, Ltd., Tokyo. Crystalline pancreatic ribonuclease and crystalline lysozyme were purchased from Worthington Biochemicals Corporation.

RESULTS

Figure 1 shows the distribution of the three major RNA components which appear when E. coli ^{32}P-RNA is prepared as described under "Experimental Procedure," and fractionated by sucrose gradient centrifugation. Each RNA component contains ultraviolet-absorbing material and acid-precipitable radioactivity. The close correspondence of the two profiles throughout the gradient suggests the absence of "unstable" RNA. Each labeled component is completely sensi-

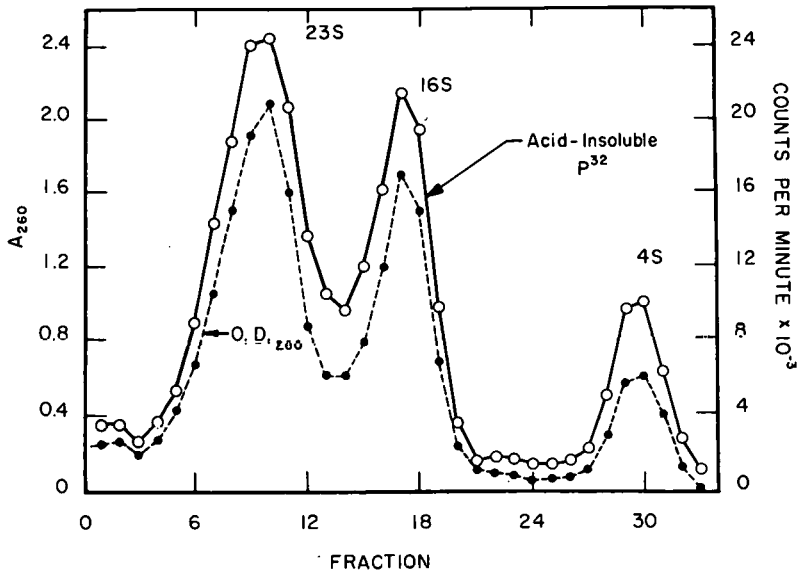

Figure 1. Sucrose gradient centrifugation of E. coli RNA. The RNA, isolated as described under "Experimental Procedure," was layered on a 30-ml linear sucrose gradient (3 to 20%), containing 0.10 M NaCl and 0.01 M Tris-HCl, pH 7.5, and centrifuged at 25,000 r.p.m. for 19 hours at 8° in an SW-25 rotor in the model L Spinco ultracentrifuge. Fractions were collected from the bottom of the tube and analyzed for acid-precipitable radioactivity and absorbance at 260 mμ.

tive to alkali and ribonuclease digestion, and bands in a region distinctly separate from DNA when subjected to cesium sulfate equilibrium centrifugation (Figure 2A).

When E. coli 23 S ^{32}P-RNA is annealed with T7 cRNA (prepared in vitro with RNA polymerase and T7 DNA) and then subjected to centrifugation in cesium sulfate, the distributions of these heterologous polyribonucleotides are not coincident (Figure 2B). Treatment of each labeled fraction with ribonuclease shows that the 23 S ^{32}P-RNA is completely degraded. In contrast, when 23 S ^{32}P-RNA is annealed with E. coli cRNA, a different situation is found. After equilibrium centrifugation, both the labeled RNA and the cRNA band in similar positions, and a significant portion of the 23 S ^{32}P-RNA now exhibits resistance to ribonuclease (Figure 2C).

The experiment in Figure 2 suggests that certain base sequences in cRNA, prepared under the direction of E. coli DNA in vitro, are complementary to the base sequences of E. coli ribosomal RNA, and that specific hybrids of 23 S RNA with cRNA are formed during annealing. Previous studies in this laboratory have shown that RNA-RNA hybrids are remarkably resistant to enzymatic degradation.[7]

Table 1 shows that both 23 S and 16 S RNA components specifically interact under annealing conditions with homologous DNA and homologous cRNA to form nuclease-resistant products. Annealing with heterologous nucleic acids does not result in the formation of ribonuclease-stable ribopolymers. The more efficient hybridization observed with E. coli cRNA, as compared with E. coli DNA, is probably due to the annealing conditions employed, which favor RNA-RNA rather than RNA-DNA interaction.

Table 2 further illustrates the specificity of annealing reactions with E. coli ribosomal RNA, and also shows hybrid formation with E. coli 4 S RNA. The complementary ribonucleic acids tested were prepared from the corresponding deoxyribonucleic acids with ribonucleic acid polymerase.

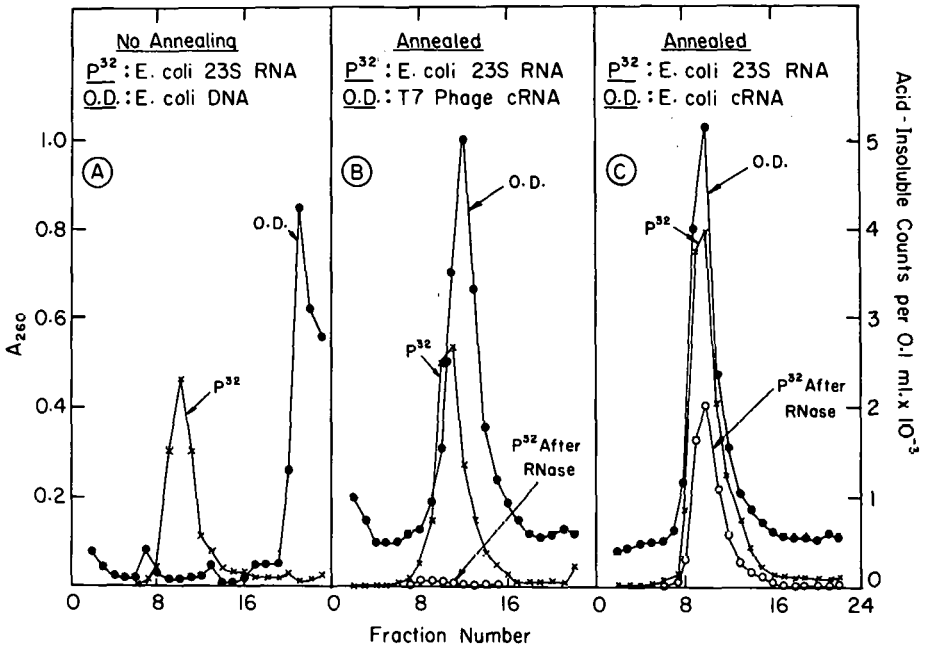

Figure 2. Cesium sulfate equilibrium centrifugation of free and hybridized RNA. Solutions were made up for centrifugation in the SW-39 rotor by mixing 1.05 ml of the nucleic acid solution in 0.05 M Tris-HCl, pH 7.5, with 2.50 ml of saturated cesium sulfate, at 25°, to give a final density of 1.610. Mineral oil (1.9 ml) was layered on the top and the solution was centrifuged at 38,000 r.p.m. for 72 hours at 25° in the model L Spinco ultracentrifuge. Fractions of approximately 0.12 ml were collected from the bottom of each tube. The fractions were analyzed for absorbance at 260 mμ and for acid-precipitable radioactivity before and after RNase treatment. A, unannealed mixture of E. coli DNA (50 μg) and E. coli 23 S ^{32}P-RNA (0.10 μg); B, mixture of T7 bacteriophage cRNA (50 μg) and E. coli 23 S ^{32}P-RNA (0.10 μg) annealed overnight at 65°; C, mixture of E. coli cRNA (50 μg) and E. coli 23 S ^{32}P-RNA (0.10 μg) annealed overnight at 65°.

It is evident that a significant amount of labeled E. coli 23 S, 16 S, and 4 S RNA, when annealed with E. coli cRNA, is converted to a ribonuclease-resistant form, when compared to annealing mixtures containing heterologous synthetic and natural ribonucleic acids. Only in the case of cRNA of Aerobacter aerogenes was the amount of ribonuclease-resistant material equal to, or slightly greater than, that formed with E. coli cRNA. Table 2 also shows that after annealing, if the reaction mixture is heated at 100°, quenched in ice, and then treated with ribonuclease, only baseline levels of acid-precipitable counts remain. Furthermore, E. coli 23 S and 16 S ribonucleic acids are not self-complementary since no resistance to ribonuclease is acquired when these ribopolymers are self-annealed.

The kinetics for the 23 S and 4 S RNA hybrid formation is illustrated in Figure 3. After 2 hours of annealing, under the conditions indicated, between 80 to 100 per cent of the hybridization process is completed. Annealing was usually carried out overnight because this was found to be convenient. Figure 4 shows that at high salt concentrations (0.2 m NaCl) optimal annealing temperatures occur in the range 70 to 80° for 23 S and 4 S RNA hybrid formation.

Although the "chase" technique, outlined under "Experimental Procedure," was designed to eliminate the presence of messenger RNA, the possibility still remained that a portion of the

Table 1

SPECIFIC HYBRID FORMATION WITH 23 S AND 16 S *E. COLI* ^{32}P-RNA

Annealing mixtures were made up as described under "Experimental Procedure." Each reaction contained 0.10 μg of either 23 S or 16 S ^{32}P-RNA and 16 μg of unlabeled DNA or RNA as indicated. After annealing overnight the mixtures were assayed for RNase-resistant counts.

Additions to *E. coli* ^{32}P-RNA annealing mixtures	RNase-resistant counts	
	23 S ^{32}P-RNA	16 S ^{32}P-RNA
	c.p.m.	c.p.m.
None (no RNase)	43,700	42,960
None	638	519
E. coli DNA	3,900	3,154
E. coli cRNA	6,186	5,573
T7 DNA	632	778
T7 cRNA	582	771
TYMV* RNA	480	548

*TYMV, turnip yellow mosaic virus.

Table 2

ANNEALING OF *E. COLI* 23 S, 16 S, AND 4 S ^{32}P-RNA WITH VARIOUS POLYRIBONUCLEOTIDE PREPARATIONS

Annealing mixtures, made up as described under "Experimental Procedure," contained 0.05 μg of either 23 S, 16 S, or 4 S *E. coli* ^{32}P-RNA and 20 μg of a specified RNA as indicated. The mixtures were annealed overnight and assayed for RNase-resistant counts.

RNA additions to annealing mixtures	RNase-resistant counts after annealing with *E. coli* RNAs		
	23 S ^{32}P-RNA	16 S ^{32}P-RNA	4 S ^{32}P-RNA
	c.p.m.	c.p.m.	c.p.m.
E. coli cRNA	2,543	2,265	1,993
E. coli cRNA (heated and quenched)	82		
E. coli 23 S RNA	59	82	47
E. coli 16 S RNA	80	60	69
A. aerogenes cRNA	2,963	2,727	1,849
M. lysodeikticus cRNA	65	117	60
Bacillus subtilis cRNA	63	40	49
Bacteriophage T7 cRNA	53	61	52
Calf thymus cRNA	72	57	81
Bacteriophage MSϕ2 RNA	70	59	33
None	50	122	83
None (no ribonuclease treatment)	19,784	17,961	22,570

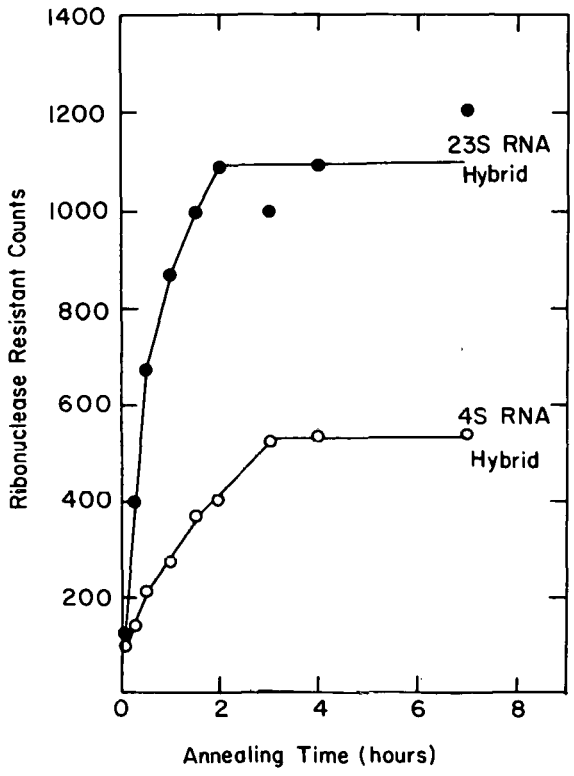

Figure 3. The formation of RNA-RNA hybrids as a function of time. E. coli cRNA (80 μg) and 0.5 μg of either 4 S or 23 S ^{32}P-RNA were mixed and adjusted to contain 0.2 M NaCl and 0.01 M Tris-HCl, pH 7.5, in a final volume of 2 ml. The mixture was heated for 5 minutes at 100°, quenched in ice, and then maintained at 65° in a water bath. Aliquots of 0.15 ml were removed at specified time intervals, quenched in ice, and assayed for RNase-resistant counts as described under "Experimental Procedure."

labeled RNA used in the above experiments contained a base composition similar to E. coli DNA. A contaminant of this character might be expected to associate readily both with E. coli DNA and with E. coli cRNA. The base compositions of the labeled 23 S and 4 S hybrids were examined to test this possibility. The base proportions of E. coli 23 S and 4 S RNA are distinctly different from those of E. coli DNA. Analysis of the labeled RNA in the 23 S and 4 S RNA-cRNA hybrids after ribonuclease treatment indicates that the base compositions are nearly identical with those of the ^{32}P-RNA used for annealing (Table 3). Other evidence, indicated below, also suggests that contamination of the labeled RNA preparations with messenger RNA cannot account for the hybrid formation observed in the experiments described here.

Tables 4, 5, and 6 show that hybrid formation for the different cytoplasmic ribonucleic acids involves different base sequences in cRNA. The amount of labeled hybrid formed with each ^{32}P-RNA can be significantly reduced only by the addition of cold cytoplasmic RNA of the same molecular species to the annealing mixture. The RNase-resistant counts formed by annealing labeled 23 S RNA with homologous cRNA are reduced by a factor comparable to the amount of nonradioactive 23 S RNA added to the reaction mixture prior to annealing (Table 4). The other molecular species of E. coli RNA (16 S and 4 S), as well as heterologous tobacco mosaic virus RNA, have little or no effect on the amount of labeled hybrid formed.

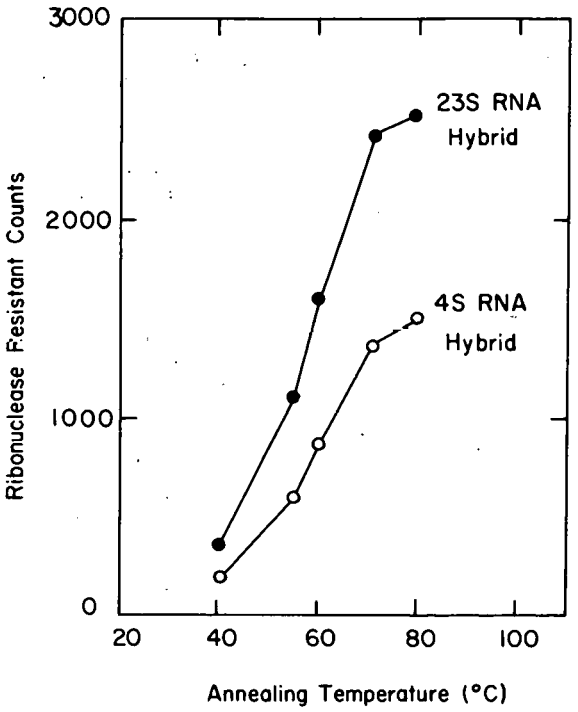

Figure 4. The effect of temperature on RNA-RNA hybrid formation. Annealing mixtures were made up as described under "Experimental Procedure." Each mixture contained 16 μg of E. coli cRNA and 0.04 μg of either E. coli 4 S or 23 S ^{32}P-RNA. Annealing was carried out overnight at the specified temperatures. Hybrid formation was determined by the RNase assay technique described under "Experimental Procedure."

When 16 S RNA is annealed with cRNA, the expected dilution of RNase-resistant hybrid is obtained only with the addition of nonradioactive 16 S RNA (Table 5). The slight reduction in counts observed with the addition of 23 S RNA is probably due to some 16 S material present in the 23 S RNA fraction used. Annealing experiments with E. coli 4 S RNA and E. coli cRNA gave similar results (Table 6). The specific dilution of labeled 23 S, 16 S, and 4 S RNA makes it unlikely that contaminating ^{32}P-messenger RNA accounts for hybrid formation, since this component would not compete for the same sites on cRNA as the 23 S, 16 S, and 4 S diluents. These data support the notions that cytoplasmic ribonucleic acids are synthesized on DNA templates and that separate cistrons code for 16 S and 23 S ribosomal RNA.

For a given quantity of cRNA, there should be a limited number of sites available for association with each cytoplasmic RNA component. The level at which saturation of these sites occurs can be determined by annealing with increasing amounts of labeled cytoplasmic RNA until a plateau in ribonuclease-resistant counts is reached. Figure 5A demonstrates the saturation plateau for two levels of E. coli cRNA determined by annealing with increasing amounts of E. coli 23 S ^{32}P-RNA. The level of saturation is directly proportional to the amount of cRNA used, while the ratio of ribonuclease-resistant counts to cRNA employed remains constant. Saturation is reached when 0.10 to 0.12 per cent of the cRNA sequences are hybridized with 23 S ^{32}P-RNA. When saturation levels were determined for E. coli DNA, by annealing with 23 S ^{32}P-RNA in the same manner, a similar value of 0.11 per cent was found (Figure 5B).

Table 3

BASE COMPOSITION OF FREE AND HYBRIDIZED E. COLI NUCLEIC ACIDS

E. coli 23 S and 4 S ^{32}P-RNA were hybridized with E. coli cRNA by the annealing conditions outlined under "Experimental Procedure." After annealing, the mixtures were equilibrated at 37°; 10 μg of pancreatic ribonuclease per ml of annealing mixture were added and allowed to incubate for 1 hour. Carrier yeast RNA (5 mg) was added, followed quickly by the addition of 5% trichloroacetic acid, and the mixture was iced. The precipitate was sedimented and washed four times by suspension in 5% cold acid. The RNA was then subjected to alkaline hydrolysis, and base composition was determined by examining the ^{32}P-nucleotide content after nucleotide separation by paper electrophoresis as previously described.[5] Base compositions of unhybridized 23 S and 4 S ^{32}P-RNA were determined in a similar manner except that annealing and ribonuclease digestion were omitted.

Base	Base proportions				
	DNA*	23 S ^{32}P-RNA used for annealing	23 S ^{32}P-RNA hybridized with cRNA	4 S ^{32}P-RNA used for annealing	4 S ^{32}P-RNA hybridized with cRNA
	mole %	mole %	mole %	mole %	mole %
C	24.9	21.8	22.3	27.7	25.6
A	24.8	27.3	28.2	19.5	20.8
G	24.9	30.6	29.6	31.0	31.7
U (T)	25.4	20.3	19.8	21.8	21.9

*Base proportions as reported by Josse, Kaiser, and Kornberg.[30]

Table 4

SPECIFIC DILUTION OF E. COLI 23 S ^{32}P-RNA-cRNA HYBRID WITH E. COLI 23 S RNA

The conditions of annealing were as described in "Experimental Procedure." Each annealing mixture contained 16 μg of E. coli cRNA and 0.05 μg of E. coli 23 S ^{32}P-RNA. Additions of unlabeled RNA to the annealing mixtures were made as indicated. Ribonuclease-resistance counts were determined as outlined under "Experimental Procedure."

Additions to E. coli 23 S ^{32}P-RNA + cRNA annealing mixtures	Dilution factor for 23 S ^{32}P-RNA	RNase-resistant counts after annealing	
		c.p.m.	%*
None	0	3744	100
Omit cRNA	0	400	
E. coli 23 S RNA (0.10 μg)	3	1784	41
E. coli 16 S RNA (0.10 μg)	0	3460	91
E. coli 4 S RNA (0.10 μg)	0	3595	95
Tobacco mosaic virus RNA (0.10 μg)	0	3800	102

*Percentage values are corrected for base-line counts obtained when cRNA was omitted.

Table 5

THE SPECIFIC DILUTION OF E. COLI 16 S ^{32}P-RNA-cRNA HYBRID WITH E. COLI 16 S RNA

The experimental conditions were the same as indicated for Table 4 except that the annealing mixture contained 0.05 μg of E. coli 16 S ^{32}P-RNA.

Additions to E. coli 16 S ^{32}P-RNA + cRNA annealing mixtures	Dilution factor for 16 S ^{32}P-RNA	RNase-resistant counts after annealing	
		c.p.m.	%*
None	0	2605	100
Omit cRNA	0	125	
E. coli 23 S RNA (0.05 μg)	0	2200	84
E. coli 23 S RNA (0.25 μg)	0	1850	70
E. coli 16 S RNA (0.05 μg)	2	1583	59
E. coli 16 S RNA (0.25 μg)	6	740	25
E. coli 4 S RNA (0.05 μg)	0	2455	94
E. coli 4 S RNA (0.25 μg)	0	2369	91
MSϕ2 RNA (0.05 μg)	0	2106	80
MSϕ2 RNA (0.25 μg)	0	2260	86

*Percentage values are corrected for base-line counts obtained when cRNA was omitted.

Table 6

THE SPECIFIC DILUTION OF E. COLI 4 S ^{32}P-RNA-cRNA HYBRID WITH E. COLI 4 S RNA

The experimental conditions were the same as indicated for Table 4 except that the annealing mixture contained 0.04 μg of E. coli 4 S ^{32}P-RNA.

Additions to E. coli 4 S ^{32}P-RNA + cRNA annealing mixtures	Dilution factor for 4 S ^{32}P-RNA	RNase-resistant counts after annealing	
		c.p.m.	%*
None	0	804	100
Omit cRNA		100	
E. coli 4 S RNA (0.04 μg)	2	547	64
E. coli 4 S RNA (0.20 μg)	6	240	20
E. coli 16 S RNA (0.04 μg)	0	943	120
E. coli 16 S RNA (0.20 μg)	0	810	101
E. coli 23 S RNA (0.04 μg)	0	856	107
E. coli 23 S RNA (0.20 μg)	0	881	111
MSϕ2 RNA (0.04 μg)	0	843	105
MSϕ2 RNA (0.20 μg)	0	879	111

*Percentage values are corrected for base-line counts obtained when cRNA was omitted.

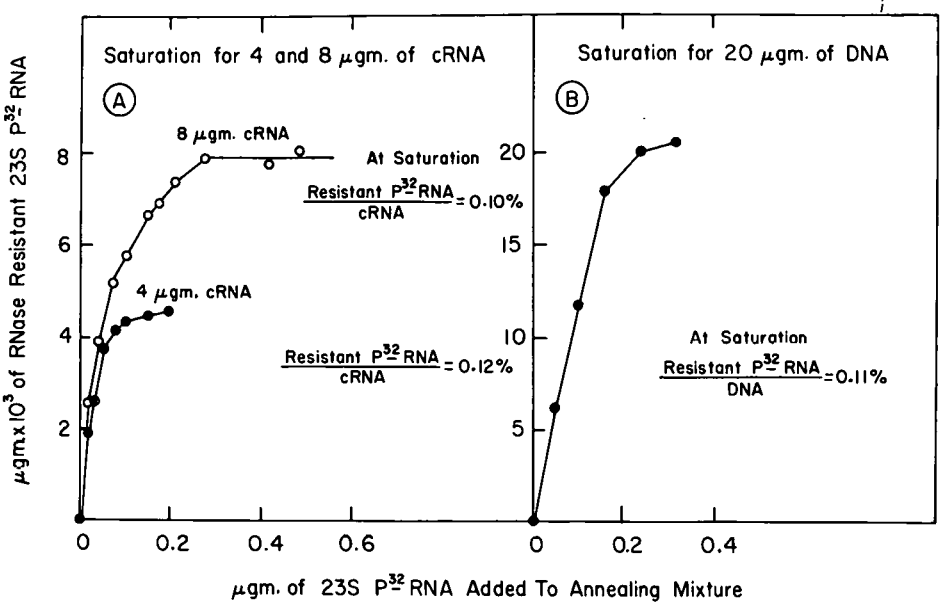

Figure 5. Saturation of E. coli DNA and cRNA with E. coli 23 S ^{32}P-RNA. A, two fixed quantities of E. coli cRNA (4 and 8 μg) were annealed with increasing amounts of 23 S ^{32}P-RNA and assayed for RNase-resistant counts as described under "Experimental Procedure"; B, the same as A except that 20 μg of E. coli DNA were used in the annealing mixture instead of cRNA.

The determination of saturation for E. coli cRNA with 4 S ^{32}P-RNA is shown in Figure 6. Saturation of cRNA occurs at 0.014 per cent indicating that only one-tenth as much cRNA is complementary to 4 S RNA as to 23 S RNA.

Previous work with a cRNA-cRNA hybrid indicated that the extent of ordered conformation could be ascertained by a study of its thermal stability.[7] When this complex is thermally dissociated it becomes susceptible to nuclease digestion. The T_m* values obtained by estimating the degree of nuclease sensitivity as a function of temperature agreed with the T_m values obtained by observing hyperchromic changes. Figure 7 shows the irreversible thermal transitions determined enzymatically for labeled 23 S and 4 S RNA-cRNA hybrids at different salt concentrations. In general, the 4 S hybrid exhibits a sharper transition than the 23 S hybrid. The transition curves shown here differ from those reported for "free" 4 S and 23 S ribonucleic acids[18] in that (a) the T_m values are considerably higher for the hybrids, and (b) the hybrids have sharper transitions.

DISCUSSION

The conclusions derived from this study rest on the assumptions that (a) the nuclease assay technique can distinguish between ordered RNA and randomly coiled RNA, and (b) RNA-RNA hybrids can be established only between RNA molecules which have reasonably large regions of sequence complementarity. A previous study of annealed cRNA indicated that it had physical properties similar to native DNA and was resistant to ribonuclease. Its ordered structure appears to protect the polyribonucleotide from ribonuclease cleavage whereas disordered RNA (heat-dena-

*The term T_m, as used here, refers to the temperature of incubation which renders 50 per cent of the RNA hybrid susceptible to ribonuclease hydrolysis.

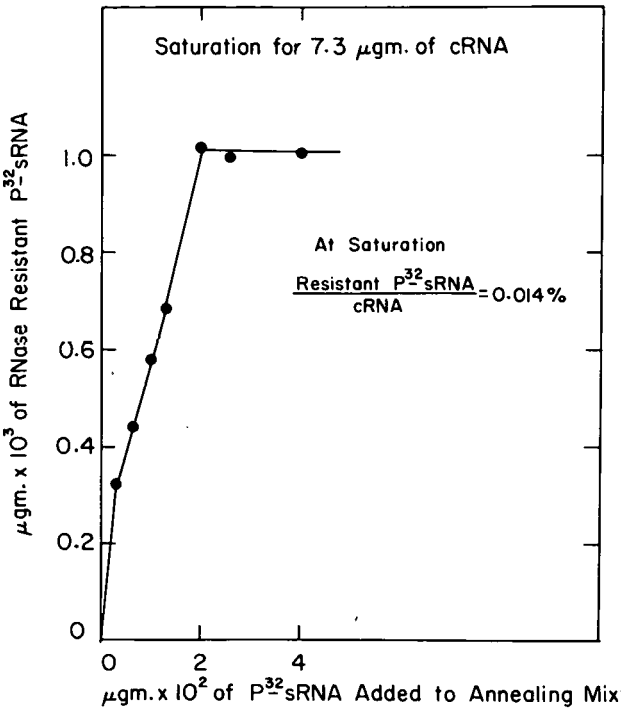

Figure 6. Saturation of E. coli cRNA with E. coli 4 S ^{32}P-RNA E. coli cRNA (7.3 μg) was annealed with increasing quantities of 4 S ^{32}P-RNA and assayed for RNase-resistant counts as described under "Experimental Procedure."

tured) readily undergoes digestion. The nuclease resistance of RNA may, therefore, be used to distinguish between randomly coiled RNA and RNA hybrids. The degree of resistance is a measure of the extent of ordered regions, which in turn depends upon sequence complementarity. The advantage of this technique over the more precise and elegant optical method for determining secondary structure, as first exploited by Doty et al.,[31] is that (a) it is simple and requires no special equipment and (b) by the use of highly labeled RNA extremely small quantities of the RNA-RNA species may be detected even in the presence of relatively large amounts of single stranded RNA molecules.

The experiments reported here show that the annealing of radioactive cytoplasmic polyribonucleotides (23 S, 16 S, and 4 S RNA) with homologous synthetic cRNA results in the formation of ribonuclease-resistant ^{32}P-RNA. Substitution of homologous cRNA by heterologous synthetic or natural ribonucleic acids in similar annealing reactions, produces no appreciable protection of the labeled RNA from enzymatic degradation. Similar findings have been reported for the annealing of ^{32}P_cytoplasmic RNA with homologous DNA.[16-18] The effectiveness of the RNase technique in distinguishing bona fide complementary RNA sequences from accidental sequence homology over short regions, or simple mechanical trapping, is illustrated by the marked specificity with which hybrid formation occurs between E. coli cRNA and homologous 23 S, 16 S, and 4 S ^{32}P-RNA (Tables 1 and 2). The negative cases found are particularly impressive.

Aerobacter cRNA showed significant association with all three species of E. coli cellular ^{32}P-RNA. These microorganisms are classified in the same family of Enterobacteriaceae.[32] Our results suggest that sequence homology occurs in ribosomal and transfer ribonucleic acids from organisms that are genetically related. Goodman and Rich have reported on cross-com-

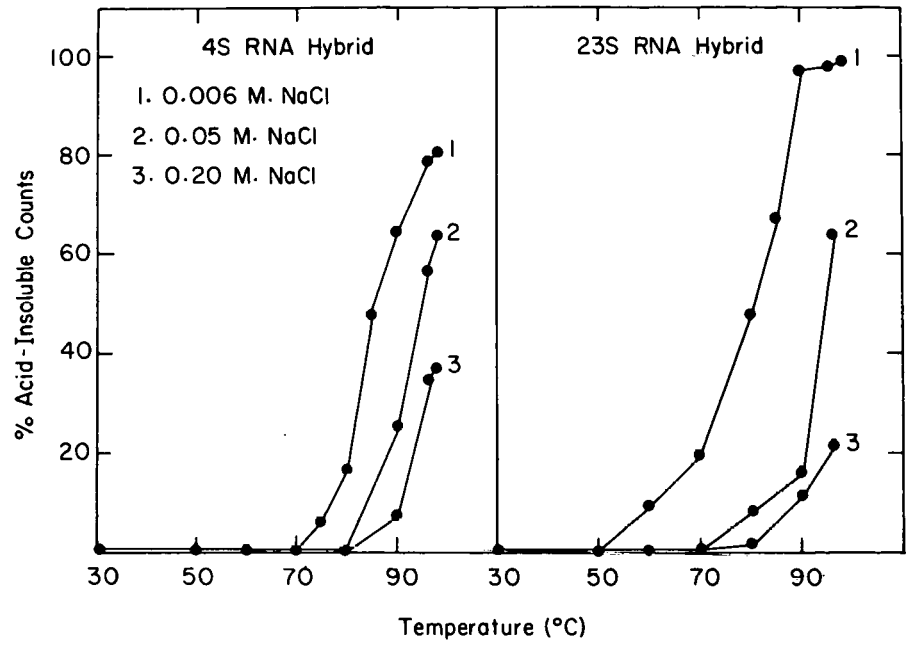

Figure 7. Irreversible thermal denaturation of 4 S and 23 S RNA hybrids. 4 S RNA hybrid: E. coli cRNA (80 μg) and E. coli 4 S ^{32}P-RNA (0.8 μg) were annealed overnight at 65° in 2 ml of 0.2 M NaCl-0.01 M Tris-HCl, pH 7.5, and then diluted to 45 ml with a solution containing 7 x 10^{-4} M Tris-HCl, pH 7.5, and 8 x 10^{-4} M Versene. This solution was divided into three separate portions and each adjusted to contain 0.006 M, 0.05 M, and 0.20 M NaCl, respectively. Samples (1 ml) were maintained at a given temperature for 15 minutes, quenched in ice, and assayed for RNase-resistant counts as described under "Experimental Procedure." 23 S RNA hybrid: the protocol for this experiment was identical with the one described above except that 0.8 μg of E. coli 23 S ^{32}P-RNA was used instead of 4 S ^{32}P-RNA.

plementarity between E. coli ^{32}P-soluble ribonucleic acid and deoxyribonucleic acid, obtained from closely related species.[17] Marmur, Falkow, and Mandel[33] have recently suggested the use of sequence complementarity in DNA as a criterion for bacterial taxonomic classification, Our results indicate that RNA-RNA hybrid formation might also serve this function.

The observation that labeled 23 S, 16 S, and 4 S RNA association with cRNA is blocked specifically and exclusively by unlabeled RNA of only the same molecular species suggests that these cytoplasmic ribonucleic acids have different base sequences and are transcribed from different regions of the DNA genome. This conclusion is in agreement with the results of Yankofsky and Spiegelman.[34]

The saturation experiments show that approximately 0.11 per cent of the nucleotide sequences in both cRNA and DNA are complementary to 23 S RNA. Saturation of cRNA with 4 S RNA occurs at 0.014 per cent. These values are in good agreement with those previously reported for DNA saturation with the same cytoplasmic RNA components.[17,18,33] The finding that the percentage of sequence complementarity to 23 S RNA is the same for cRNA and DNA implies either that sequences representative of the entire DNA molecule are replicated during cRNA synthesis, or that the values obtained were fortuitous. Repeat experiments with different preparations of cRNA give similar results. If the saturation values for cRNA are accidental, then the RNA polymerase system repeats the same mistake in independent syntheses of cRNA.

The studies on hybrid stability indicate that the RNA-RNA complexes do not readily dissociate. With increasing salt concentrations the temperatures required for the thermal denaturation also increase. In 0.20 M NaCl, the hybrids are only partially denatured even after 15 minutes of heating at 100° (Figure 5). This thermal stability of the hybrid in high salt concentrations suggests the reason for the high annealing temperature (70-80°) found to be optimal. The relative sharpness of the irreversible thermal transition profiles suggests that the RNA-RNA complexes contain a high degree of secondary structure.

A recent publication from this laboratory has shown that phage α messenger RNA is complementary to synthetic cRNA prepared in vitro with RNA polymerase and phage α DNA.[12] The present report provides additional experimental evidence which suggests that synthetic cRNA, made from bacterial DNA with RNA polymerase, is complementary to homologous naturally occurring 23 S, 16 S, and 4 S RNA. Since cRNA exhibits a high degree of self-complementarity, which is believed to result from the copying of both strands of DNA in the polymerase system, it is reasonable to conclude that, in vitro, cRNA chains are synthesized which are both complementary and identical in base sequence with the naturally occurring cytoplasmic ribonucleic acids. These results also provide evidence that ribosomal and transfer ribonucleic acids originate from the cellular activity of the DNA-directed RNA polymerase reaction, in agreement with the conclusions of Goodman and Rich[17] and Yankofsky and Spiegelman.[16]

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THE INFLUENCE OF POLYCYCLIC AROMATIC HYDROCARBONS ON BACTERIOPHAGE DEVELOPMENT*

By

W-T. Hsu, J. W. Moohr, and S. B. Weiss

Polycyclic aromatic hydrocarbons exhibit striking biological effects in mammalian organisms, e.g., they may act as carcinogens. The biochemical mechanism by which they exert this influence, however, remains obscure. While the literature on hydrocarbon action in higher organisms is extensive, attempts to demonstrate biological responses in lower forms have been largely unsuccessful. This striking difference between the effects on higher and lower forms is difficult to rationalize if one accepts those current concepts which hold that hydrocarbons alter phenotypic expression by modifying cellular nucleic acid and/or protein synthesis, either directly or indirectly. The disparity might be explained by (a) the impermeability of intact microbial organisms to the nonpolar hydrocarbons, and (b) the lack of a sufficiently sensitive response which would be readily detectable in lower forms. These considerations led to an examination of the hydrocarbon influence on bacteriophage replication in bacterial spheroplasts.

This preliminary report describes the selective inhibition of both RNA and DNA bacteriophage multiplication in Escherichia coli spheroplasts by carcinogenic polycyclic hydrocarbons. Recently, De Maeyer et al. observed that carcinogenic hydrocarbons inhibit the replication of the DNA† viruses herpes and vaccinia in rat embryo cell cultures.[1] In contrast to the work now reported, these investigators found no effect of hydrocarbons on RNA virus replication.

EXPERIMENTAL

Infectious nucleic acid. Infectious RNA was isolated by phenol extraction of MS2 phage which had been grown on E. coli K12 W1485.[2] The final MS2 RNA preparation was dissolved in 5 mM EDTA, pH 7.4, at a concentration of 3-5 mg per ml and stored frozen at -20°. Infectious lambda DNA was a gift from Dr. R. Mackal and infectious ΦX174 DNA was a gift from Drs. S. Spiegelman and J. Grunau.

Preparation of E. coli spheroplasts. E. coli spheroplasts were prepared essentially by the method of Guthrie and Sinsheimer.[3] E. coli K12W1485 (obtained from Dr. B. Strauss) was grown overnight. From this culture, 0.50 ml was transferred into 10 ml of 3XD medium[4] and grown to a concentration of 5×10^8 cells per ml. Four ml of this culture was transferred into 50 ml of 3XD medium and grown once more to the same concentration. All of the above growth steps were at 37°. Approximately 35 ml of this final culture was transferred to a conical centrifuge tube, sedimented for 15 min at 5000 x g at room temperature, and the pellet resuspended in 0.70 ml of 50 per cent sucrose. To this cell suspension were added 0.34 ml of sterile 30 per cent bovine serum albumin (Armour Pharmaceutical Co.), 0.08 ml of a 1 mg per ml lysozyme solution

*This paper appeared in Proc. Nat. Acad. Sci. U. S., 53:517, 1965.

†The following abbreviations are used: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; EDTA, disodium ethylenediaminetetraacetate; DMF, dimethylformamide; PFU, plaque forming unit; DMBA, dimethylbenz [a] anthracene; MBA, methylbenz [a] anthracene.

(Worthington Biochemical Corp.), and 0.04 ml of 0.12 M EDTA pH 7.4 mixed and transferred quantitatively into 20 ml of PAM medium.[3] The PAM medium used here was modified to contain no peptone. After incubating this mixture for 15 min at 37°, 0.40 ml of 1 M $MgSo_4$ was added to the cell suspension, which was then allowed to stand at room temperature for 1 hr before use.

Assay for infectious nucleic acid activity. The stock solutions of infectious nucleic acid were diluted with 2.5 mM EDTA, pH 7.4, so that 0.50 ml would give a few hundred plaque forming units (PFU) in the assay system. Stored viral nucleic acids, especially MS2 RNA, slowly decrease in infectivity with age. A 1.1-ml infectious mixture contained 0.50 ml of the diluted nucleic acid solution and 0.50 ml of the spheroplast preparation (treated or nontreated with hydrocarbon as described below). Infectious mixtures of more than 1.1 ml were prepared in the same manner but with proportionately more material. The nucleic acid-spheroplast mixture was incubated for 10 min at 30° (unless otherwise indicated) and assayed for PFU on the appropriate indicator bacteria by the agar layer procedure of Adams.[5] The indicator bacteria used were E. coli K12W1485 for MS2 phage assay, and E. coli C (obtained from Dr. R. Sinsheimer) for ΦX174 phage assay.

Hydrocarbon addition to the assay system. Stock solutions of the various hydrocarbons were prepared in dimethyl formamide (DMF). Aliquots of these solutions were diluted with water so that the desired quantity in a volume of 0.10 ml, could be added to 0.50 ml of spheroplasts. The concentration of DMF in the spheroplast mixture at this point was 0.8 per cent (v/v), and did not alter the efficiency of plaque formation. Control spheroplasts contained the same quantity of DMF but no hydrocarbons. The spheroplast-hydrocarbon mixtures and the spheroplast controls were then incubated for 50 min at 30° prior to mixing with the infectious nucleic acid. This procedure was routine except where otherwise stated. All vessels were wrapped in aluminum foil to exclude light at each of the steps described and this procedure was also followed for plating onto the agar Petri dishes for phage assay. For most of the stock hydrocarbon solutions tested, dilution with water resulted in a very fine suspension of the aromatics. The hydrocarbons were generously donated by Dr. Charles B. Huggins.

Assays for the DNA-directed synthesis of RNA and DNA in lysed cells were performed by the methods of Nakamoto et al.[6] and Lehman et al.,[7] respectively.

RESULTS

Nucleic acid preparations from a number of DNA- and RNA-containing bacteriophages are infectious for bacterial spheroplasts.[3,8-12] Although infection with viral nucleic acid is far less efficient than infection with the whole virus, the nucleic acid-spheroplast system is sufficiently sensitive so that 0.01-0.10 mμg of MS2 nucleic acid gives rise to approximately one plaque forming unit.

In the course of examining certain properties of the infectious MS2 RNA-spheroplast system, we observed that relatively low concentrations of the carcinogen 7,12-Dimethylbenz [a] anthracene (7,12-DMBA) were potent inhibitors of plaque formation. This inhibition was readily reproducible. When other closely related hydrocarbons were tested in the system, it became obvious that a correlation existed between the observed carcinogenic activity of a compound and its ability to inhibit plaque formation (Tables 1 and 2). Exceptions to this were 1,12-DMBA, which is a potent virus inhibitor but does not give rise to sarcomas in rats,[13] and two azo compounds, 4-dimethylaminoazobenzene and its 3-methyl derivative, which have no effect in the infectious nucleic acid

Table 1

EFFECT OF VARIOUS HYDROCARBONS ON MS2 PLAQUE YIELD

Expt. no.	Hydrocarbon		PFU per ml	Carcinogenic activity*
	Type	Amount μg		
1	None	—	238	
	7,12-DMBA	20	32	+
		5	77	
	3,9-DMBA	20	151	-
		5	185	
	6,8-DMBA	20	38	+
		5	150	
	1,12-DMBA	20	24	-
		5	78	
2	None	—	280	
	7,12-DMBA	10	44	+
	3,9-DMBA	10	288	-
	6,8-DMBA	10	97	+
	1,12-DMBA	10	12	-

*+ produces sarcoma when 2.5 mg of hydrocarbon in 0.5 ml of sesame oil is injected in muscle of rat.[13] The final volume of the infectious mixture was 1.1 ml. MS2 RNA (50 mμg) served as the infectious agent. The amount of each hydrocarbon used is indicated above; they were prepared for testing as described under Experimental. The entire contents of the mixture was used for the PFU assay and each figure represents the average of duplicate assays.

system and are carcinogenic. The latter compounds produce sarcomas in the liver only and then under special conditions such as prolonged feeding.

For maximum inhibition of virus production, we have found it necessary to preincubate the bacterial spheroplasts with the hydrocarbon prior to infection. Under these conditions, 7,12-DMBA inhibits plaque formation 90 per cent or more at concentrations of approximately 10 μg per ml of infectious mixture (Figure 1). If the spheroplast-preincubation procedure is omitted and the hydrocarbon is added simultaneously with the infectious RNA to bacterial spheroplasts, the inhibition rarely exceeds 60 per cent even at concentrations of hydrocarbon greater than 20 μg per ml of infectious mixture. Preincubation of the viral nucleic acid with the hydrocarbon also results in incomplete inhibition.

The inhibition of bacteriophage growth by carcinogenic polycyclic aromatic hydrocarbons is not exclusive for RNA viruses alone. Table 3 shows that certain hydrocarbons depress plaque formation when bacterial spheroplasts are infected with ΦX174 DNA. Here too, one observes some correlation between hydrocarbons which exhibit carcinogenic activity and those which inhibit plaque formation; however, as with MS2 RNA, certain exceptions were found (1,12-DMBA and 6-MBA).

The possibility was considered that the active hydrocarbon might exert its inhibitory effect

Table 2

EFFECT OF VARIOUS HYDROCARBONS ON MS2 PLAQUE YIELD

Expt. no.	Hydrocarbon		PFU per ml	Carcinogenic activity*
	Type	Amount μg		
1	None	—	261	
	7,12-DMBA	20	40	+
	4-MBA	20	289	-
	6-MBA	20	67	+
	5-MBA	20	230	-
2	None	—	418	
	7,12-DMBA	20	48	+
	Benz [a] anthracene	20	451	-
3	None	—	138	
	7,12-DMBA	20	35	+
	Chrysene	20	128	-
	3-Aminochrysene	20	62	+
	Benzo [a] pyrene	20	55	+

*+ produces sarcoma when 2.5 mg of hydrocarbon in 0.5 ml of sesame oil is injected in muscle of rat.[13] The assay condition was identical to that described in Table 1, except that the quantity of infectious MS2 RNA used in Experiment 1, 2 and 3 was 50, 80 and 25 mμg respectively.

by preventing the viral nucleic acid from penetrating the cell. To determine this, 7,12-DMBA was added to the infectious system at various intervals after spheroplast exposure to MS2 RNA. To insure against the presence of extracellular infectious RNA at the time of hydrocarbon addition, pancreatic ribonuclease was introduced into the mixture prior to the introduction of 7,12-DMBA. Table 4 gives the results of such an experiment. When 2 μg of RNase is added almost simultaneously with infectious MS2 RNA to spheroplasts (zero time) only 1 per cent of the potential PFU survives the nuclease treatment. However, addition of RNase 10 min or more after spheroplast exposure to the infectious RNA results in no significant alteration of plaque formation. The infectious process therefore is complete within 10 min after RNA addition to the bacterial spheroplasts.[14] Table 4 shows that the inhibitory effect of 7,12-DMBA is demonstrable even when added 45 min after infection has taken place, and that the later the time of hydrocarbon addition, the greater the number of infective centers, which approaches the plaque yield found in the absence of hydrocarbon. It appears, therefore, that the hydrocarbon exerts its effect on the production of virus, and not on the entry of nucleic acid into the cell. This experiment also suggests that the hydrocarbon inhibits some process early in viral development prior to whole phage formation.

To eliminate the possibility that the hydrocarbon might prevent the lysis of the bacterial spheroplast and hence the detection of newly formed phage, hydrocarbon-treated and nontreated infected cells were artificially lysed at various intervals and assayed with intact bacteria for the appearance of whole virus (Figure 2). In this system, a leveling off of phage production may

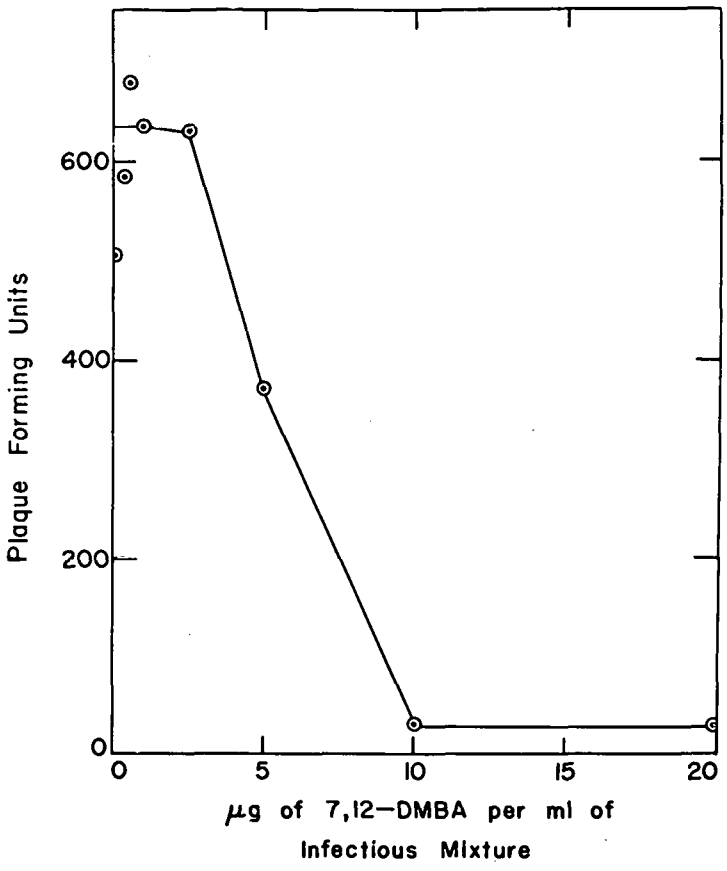

Figure 1. The effect of 7,12-DMBA concentration on plaque yield. The infectious system was 1.1 ml, and 100 mμg of MS2 RNA was used. The procedure was similar to that described in Table 1 except that various amounts of 7,12-DMBA were used for the pretreatment of E. coli spheroplasts. The amount of solvent was constant at each concentration. Each point represents the average value of duplicate assays.

may be observed between 210-240 min after infection, in both the hydrocarbon-treated and nontreated bacterial cells, but the final titer of the nontreated cells is approximately 10-fold higher than the cells exposed to 7,12-DMBA. Furthermore, whereas the nontreated cells demonstrate whole phage synthesis 90 min after infection, production of whole phage is delayed in the 7,12-DMBA-treated cells until 180 min. This experiment suggests that the hydrocarbon phenomenon is not a result of cell-lysis inhibition, but rather an inhibition in the rate and extent of whole phage replication.

As yet, we have been unable to detect any impairment of nucleic acid or protein synthesis in uninfected bacterial spheroplasts by 7,12-DMBA. Spheroplasts which have been pretreated with hydrocarbon show no significant difference in their rate of C^{14}-uracil or C^{14}-amino acid incorporation into acid-insoluble material when compared to non-hydrocarbon treated cells (Figure 3). The similarity in the rates of labeled substrate incorporation suggests that the hydrocarbon is not inducing premature cell lysis. Furthermore, lysed spheroplasts, with or without hydrocarbon pretreatment, show no difference in their ability to carry out the DNA-directed synthesis of DNA or the DNA-directed synthesis of RNA. These same preparations, however,

Table 3

EFFECT OF VARIOUS HYDROCARBONS ON ΦX174 PLAQUE YIELD

Hydrocarbon		PFU	Carcinogenic activity*
Type	Amount μg		
None	—	563	
7,12-DMBA	10	38	+
3,9-DMBA	10	517	–
6,8-DMBA	10	44	+
1,12-DMBA	10	27	–
4-MBA	10	488	–
6-MBA	10	552	+
5-MBA	10	586	–
Benzo [a] pyrene	10	236	+

*+ produces sarcoma when 2.5 mg of hydrocarbon in 0.5 ml of sesame oil is injected in muscle of rat.[13] The assay condition was identical with that described in Table 1, except that 0.10 mμg of ΦX174 DNA served as the infectious agent.

Table 4

EFFECT OF 7,12-DMBA ON MS2 PLAQUE YIELD WHEN ADDED AT VARIOUS TIMES AFTER INFECTION

Time of RNase addition after infection (min)	Addition of 7,12-DMBA (5 min after RNase)	PFU	% PFU production
No RNase	–	275	100
0	–	4	1
	+	4	1
10	–	262	95
	+	100	36
20	–	300	109
	+	124	45
40	–	292	106
	+	172	62

The infectious mixture was 1.0 ml, and 50 mμg of MS2 RNA was used. Pancreatic ribonuclease (2 μg) was added at various times to the spheroplast mixture either simultaneously (zero time) or after MS2 RNA addition. Twenty micrograms of 7,12-DMBA, in 0.10 ml of diluted solvent was added to the appropriate vessels 5 minutes after RNase treatment. The control vessels received 0.10 ml of diluted solvent but without hydrocarbon. After 10 min exposure to the hydrocarbon the entire mixture was assayed for infective centers. The values presented are the average of duplicate assays.

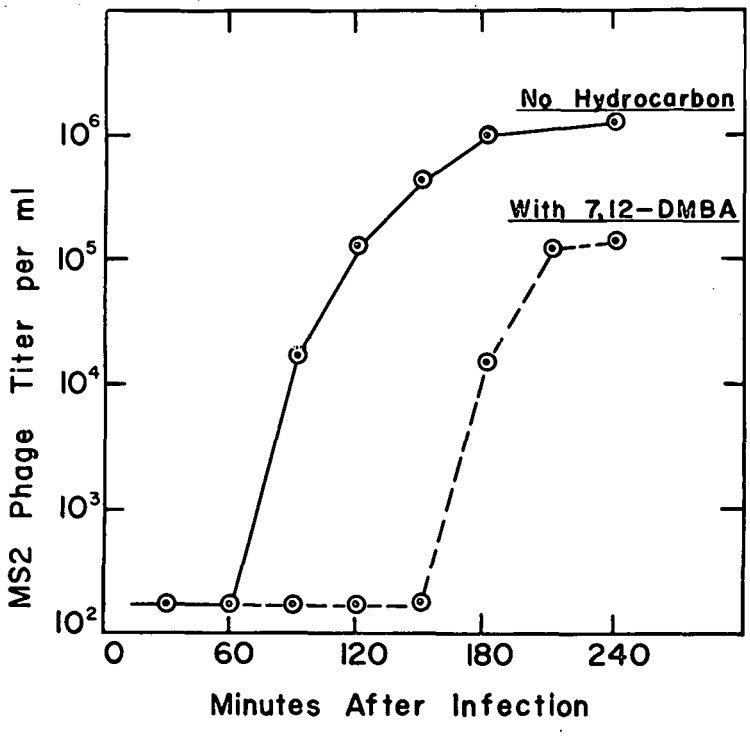

Figure 2. The effect of 7,12-DMBA on MS2 phage development. The infectious mixture was 22 ml, and 1 μg of MS2 RNA was used. Spheroplasts, with or without pretreatment with 7,12-DMBA (20 μg per ml of infectious mixture), were exposed to MS2 RNA and at various intervals 0.10 ml of the mixture was added to 0.4 ml of lysing medium containing 4 drops of $CHCl_3$. The lysing medium contained 0.01 ml of 0.1 M KCN; 0.05 ml of lysozyme (2 mg per ml); 0.05 ml of 0.50 M EDTA; and 0.02 ml of 1.0 M Tris·HCl (pH 8.1), adjusted to 1 ml with PA medium.[3] After standing for 30 min at 30°, appropriate dilutions were made to assay for the presence of whole phage.

are quite sensitive to actinomycin D for labeled ribonucleotide incorporation into RNA.[15] Using highly purified preparations of Micrococcus lysodeikticus RNA polymerase, hydrocarbon additions to reaction mixtures show no effect on the polymerization of ribonucleotides.

Indirect evidence suggests that the phenomenon described here is not a result of host-induced enzyme synthesis which subsequently blocks viral replication. In the presence of actinomycin D (20 μg per ml of infectious mixture), RNA synthesis in E. coli spheroplasts is inhibited 80-90 per cent, but MS2 phage production is not impaired.[16] However, 7,12-DMBA still exerts its inhibitory effect on phage production when actinomycin D treated cells serve as host.

DISCUSSION

The present study indicates that polycyclic aromatic hydrocarbons do exert some biological influence on the replication of certain bacterial viruses. The inhibitory effect observed for the replication of MS2 and ΦX174 viruses may not be general for all bacteriophage. Preliminary experiments with infectious lambda DNA showed that there was an increase in plaque formation of lambda virus, rather than a decrease, when E. coli spheroplasts were pre-exposed to polycyclic

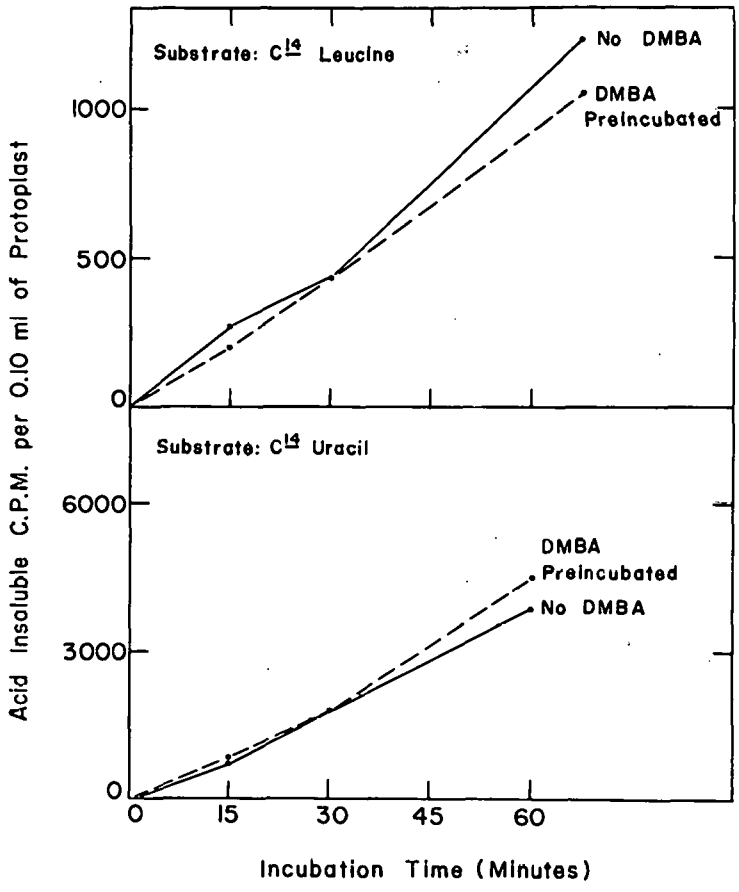

Figure 3. The effect of 7,12-DMBA on C^{14}-uracil and C^{14}-leucine incorporation into the acid-precipitable fraction of E. coli spheroplasts. To 0.50 ml of E. coli spheroplasts, with or without hydrocarbon pretreatment, was added 0.50 ml of 5 mM EDTA and either 0.02 ml of 2-C^{14}-uracil (1.3 mM, 65 mc per mM) or 0.05 ml of 1-C^{14}-leucine (5 mM, 12 mc per mM). The mixtures were incubated at 30° and at various times 0.10 ml aliquots were transferred into 2.5 ml of 5 per cent trichloroacetic acid. The C^{14}-uracil aliquots were prepared for counting by washing on millipore filter as previously described.[13] The C^{14}-leucine samples were subjected to 15 min heating at 90°, in 5 per cent trichloroacetic acid, prior to millipore filter washing for count determination.

hydrocarbons. Although some exceptions were found, both with infectious MS2 RNA and ΦX174 DNA, the ability of hydrocarbons to inhibit viral growth exhibits an impressive correlation with their carcinogenic activity. Several points may be made about the hydrocarbon influence on viral replication: (1) the hydrocarbon effect appears to result neither from the alteration of host DNA, RNA, or protein synthesis, nor from the induction of an inhibitory host enzyme(s), (2) the hydrocarbon does not inhibit the adsorption of viral nucleic acid nor the release of newly formed virus, (3) the hydrocarbon has no effect on virus production when intact bacteria are infected with whole phage, (4) mammalian cells are not alone in their biological response to hydrocarbon

administration, (5) it is by no means certain that the two phenomena elicited by the same chemical agents, namely carcinogenic action and viral growth responses, are related to one another.

In recent years, it has been suggested that certain hydrocarbons evoke their biological effect at some level involving nucleic acids.[17-22] Hydrocarbon-induced changes in animal tissue have been blocked by prior treatment with actinomycin D.[18] Several workers have presented evidence on the binding and "solubilization" of polycyclic hydrocarbons by nucleic acids.[19-21] It has been reported that 7,12-DMBA selectively damages mammalian cells undergoing active DNA synthesis and depresses H^3-thymidine incorporation into the DNA of these same cells.[22] It is not clear whether the effects described in these reports are directly related to the carcinogenic action of polycyclic hydrocarbons.

De Maeyer et al. have drawn analogies between the action of carcinogenic hydrocarbons and actinomycin D.[1] These investigators concluded that the action of 7,12-DMBA and benzo [a] pyrene is similar to actinomycin D since both inhibit interferon synthesis in virus infected tissue cultures.[23,24] More recently they have shown that carcinogenic hydrocarbons inhibit the development of only the DNA-containing viruses in mammalian cell cultures, hence, polycyclic hydrocarbons exhibit an actinomycin D-like effect.[1]

The experiments presented here confirm the observations of De Maeyer et al. that carcinogenic hydrocarbons inhibit the replication of certain viruses, but our evidence also suggests that the mechanism of viral inhibition differs from the action of actinomycin D in that (1) actinomycin D does not interfere with the replication of the RNA virus, MS2; hydrocarbons do, (2) hydrocarbon treatment of E. coli spheroplasts, which depresses viral replication, has little or no effect on E. coli spheroplast synthesis of RNA; actinomycin treatment of these same cells inhibits RNA synthesis markedly, and (3) hydrocarbons do not interfere with the DNA-directed synthesis of RNA, in vitro, using highly purified DNA and RNA polymerase; actinomycin D does.

At present, the mechanism of viral growth inhibition by polycyclic hydrocarbons is obscure. Whether hydrocarbon-induced viral inhibition and carcinogenesis are related by the action of the aromatics at the same basic biochemical level also remains obscure. While the problem of carcinogenic induction by hydrocarbons is quite complex, it is our hope that the mechanism of virus inhibition may prove simpler to approach.

ACKNOWLEDGMENT

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STEROID FEVER AND INFLAMMATION. STUDIES WITH 11-KETOPREGNANOLONE IN MAN*

By

P. B. Glickman,† R. H. Palmer, and A. Kappas

A number of 5β-H steroids derived from the endogenous metabolic transformations of cholesterol and gonadal and adrenocortical hormones have been shown to evoke intense pyrogenic reactions when injected into humans. The structural basis and species specificity of this form of biological activity have been described in previous communications from this laboratory.[1-7]

The present report examines in detail certain other aspects of steroid pyrogen action, utilizing 11-ketopregnanolone as a chemical prototype of this class of fever-producing agents. This C21 5β-H steroid was selected for use in this study because of its potency, its physiologic origin, and its ready availability in large amounts and in pure form through chemical synthesis. The characteristics of the thermogenic and inflammatory responses induced by this compound may, on the basis of extensive comparisons made with structurally related steroids, be considered to typify those induced generally by appropriate administration of neutral steroid pyrogens to man.

METHODS AND RESULTS

The 11-ketopregnanolone used to provoke fever in these studies was synthetically prepared, and repeatedly purified until its spectroscopic, chromatographic, and physical characteristics, were equal to the best samples available. During the course of this investigation several different preparations of steroid were tested, and all gave equivalent pyrogenic responses in man while none provoked fever in large doses in a wide variety of experimental animals, including rabbits, dogs, cats and monkeys.‡

For intramuscular injection, the crystalline compound was dissolved in a benzyl alcohol-sesame oil (15:85) or propylene glycol solvent vehicle in a concentration of 25 mg/ml. For intravenous administration the steroid was dissolved in absolute ethanol in approximately the same concentration. Prolonged intravenous infusions were made over varying periods of time by means of a constant infusion pump (Harvard Apparatus Co., Model 600-900). Wherever possible, equipment used in the preparation or administration of steroid, such as syringes, needles, tubes, etc., was heated at 200° C for two or more hours immediately before use to destroy bacterial pyrogen contamination. If this was not possible (as in the case of the short lengths of polyethylene tubing used for intravenous infusions) equipment was sterilized chemically and washed with large volumes of pyrogen-free water immediately before use. Solvent vehicles were tested concurrently and shown to be free of pyrogenic activity in man.

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‡ 11-ketopregnanolone as well as structurally related steroid pyrogens have also been shown to be non-pyrogenic in chimpanzees, when administered in amounts much larger than those which consistently provoke fever in humans (Riopelle, A. and Kappas, A.: unpublished observations).

All subjects used in this investigation were adult volunteer hospital patients with ages ranging from 23 to 75 years. The sex distribution was approximately equal in most studies, although no sex difference in the response to steroid pyrogen action was observed. Clinical diagnoses varied, although the majority had incurable malignancy. Most were reasonably well nourished, however, and ambulatory. All were housed on a metabolic ward during these studies. Radiotherapy was being given to some patients during the tolerance studies, as part of a concurrent investigation on the influence of temperature on radiosensitivity of tumors.[8]

Intramuscular Injection of Steroid Pyrogen

Dose response study. The relationship between steroid pyrogen dose and intensity of fever provoked was examined as follows:

Total dose 5 mg. Twelve injections were made in 12 subjects of which six resulted in fever. Average temperature increase in the subjects developing fever was 2.5° F (range 2.0 to 3.2° F); average lag time of fever was 6-1/2 hours (range 5 to 8 hours); average temperature peak was reached at 11 hours (range 9 to 16 hours); and average time required for temperature to return to normal was 20 hours (range 15 to 24 hours).

All 12 subjects used in this study were included in the two groups described below and all, including those unresponsive to 5 mg of steroid, developed pyrogenic reactions to 10 mg or more of the compound.

Total dose 10 mg. Thirty-eight injections at this dose were made in 14 subjects, of which 36 resulted in fever. Included among these 14 subjects were two who received repeated daily injections of steroid for tolerance studies, and two who received several injections at less frequent intervals for other purposes. Since pyrogenic responses to repeated injections of steroid were comparable in each subject, all were included in the calculated values following.

Average temperature increase in the 36 injections resulting in fever was 3.6° F (range 1.8 to 5.6° F); average lag time was 7-1/2 hours (range 6 to 10 hours); average temperature peak was reached at 11 hours (average 7 to 16 hours); and average time required for temperature to return to normal was 21 hours (range 16 to 24 hours).

Total dose 25 mg. One hundred and thirty-nine injections at this dose were made in 31 patients, of which 136 resulted in fever. Two of the unsuccessful injections were made in one subject who developed consistent pyrogenic reactions to 37.5 mg of steroid; the other injection was made in one subject who developed fever only after injections of 200 mg of steroid were made. Included among the 136 pyrogenic injections were 88 given to 11 subjects participating in tolerance studies.

Average temperature increase in the 136 febrile reactions was 3.8° F (range 2.0 to 6.5° F); average lag time was 6-1/2 hours (range 4 to 10 hours); average temperature peak was reached at 10 hours (range 6 to 14 hours); and average time required for temperature to return to normal was 22 hours (range 16 to 28 hours).

Figure 1 depicts the dose-response fever curves in 4 subjects receiving 5, 10 and 25 mg of steroid. Figure 2 indicates the consistency and character of the temperature response of 20 subjects receiving 25 mg of steroid pyrogen. This figure does not depict the small secondary rises in temperature which occurred in some patients on the second day after injection of steroid pyrogen.

Tolerance studies. The temperature response to repeated injection of steroid pyrogen was examined with reference to the possible development of tolerance[9,10] in 15 subjects as follows:

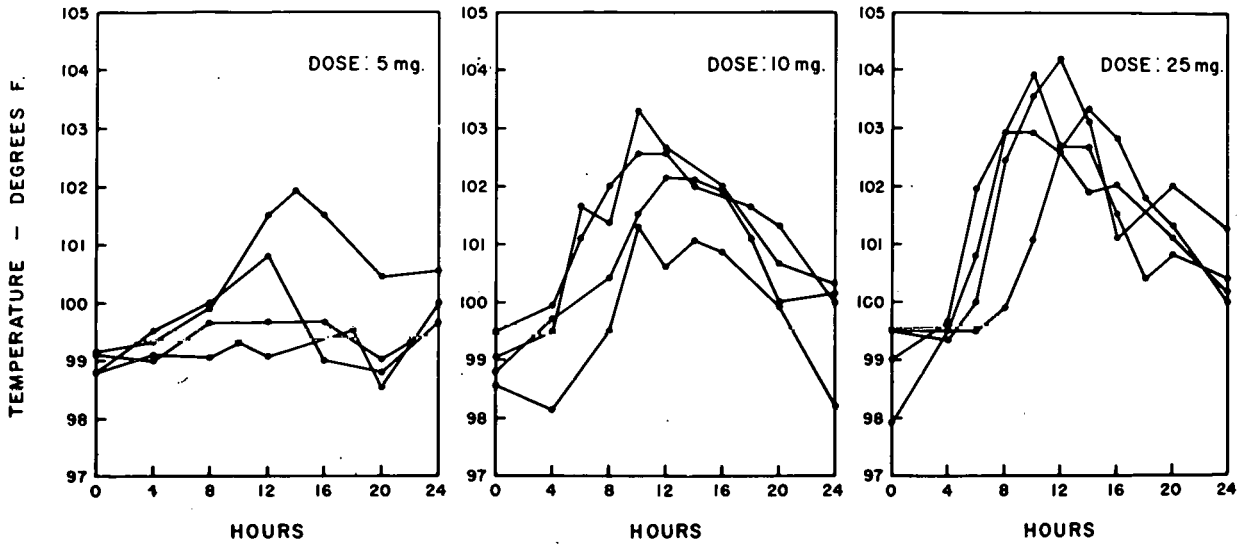

Figure 1. Dose-response fever curves in 4 subjects receiving 5, 10 and 25 mg injections of 11-ketopregnanolone I.M.

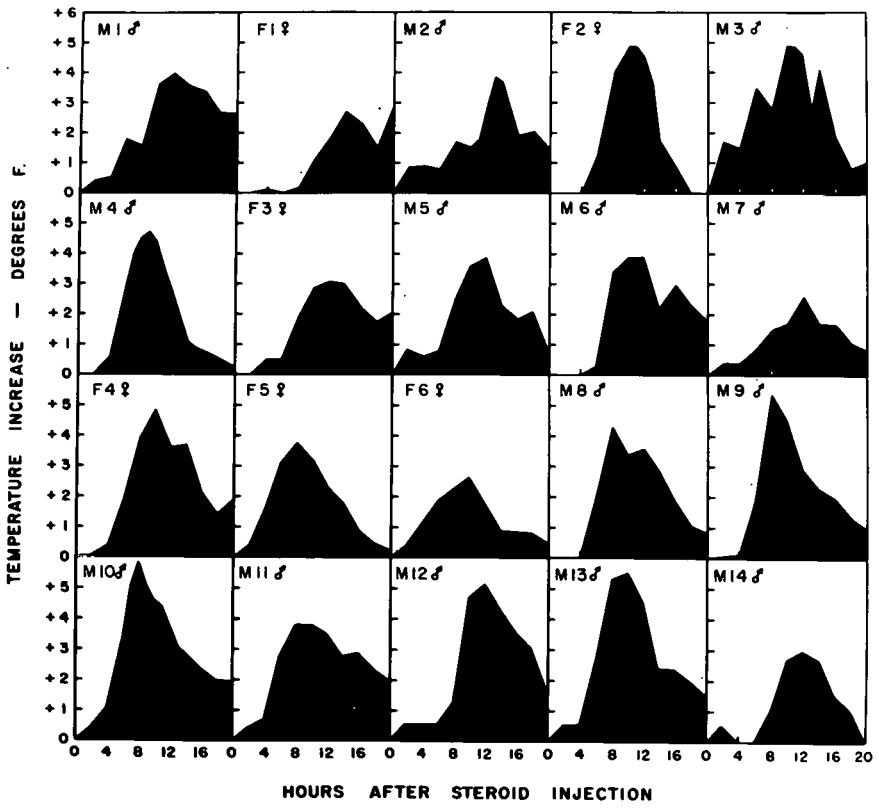

Figure 2. Representative fever curves in 20 different subjects receiving injections of 25 mg of 11-ketopregnanolone I.M.

two subjects received 10 mg injections of steroid daily for 10 days; 5 received 25 mg injections per day for 4 days; one received 25 mg per day for 5 days; one received 25 mg per day for 8 days; one received 25 mg per day for 12 days; one received 25 mg per day for 15 days; one received 25 mg per day for 20 days; one received 25 mg per day for 24 days and two received 37.5 mg per day for 7 days.

Repeated daily injection of steroid pyrogen was associated with a slight decrease in the latency period of fever production in some subjects, but the character and intensity of the thermogenic response was not otherwise altered and no tendency to the development of tolerance was observed. Figure 3 demonstrates the pyrogenic responses on various days of the tolerance studies in four subjects receiving daily 25 mg injections of steroid. Pyrogenic responses to repeated daily injection of lesser amounts of 11-ketopregnanolone have been described elsewhere.[4]

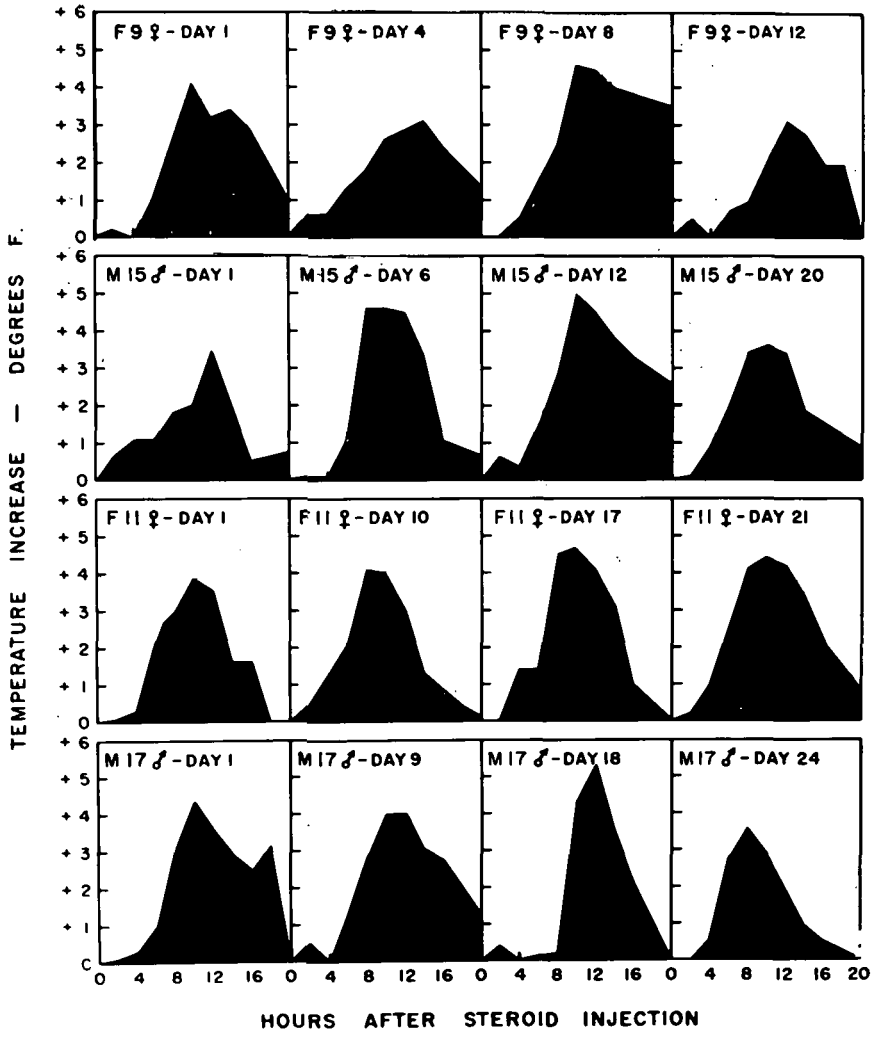

Figure 3. Pyrogenic responses in 4 subjects receiving repeated daily I.M. injections of 11-ketopregnanolone.

Intravenous injection of steroid pyrogen. A total of 28 injections was made in 14 subjects and of these injections 14 resulted in fever. The 28 steroid infusions included 8 in a dose of 25

mg in 4 subjects; one in a dose of 37.5 mg; 15 in a dose of 50 mg in 10 subjects and 4 in a dose of 75 mg in 3 subjects. Infusion times varied from 30 to 270 minutes. Three of the 25 mg injections, the 37.5 mg infusion, 8 of the 50 mg injections, and two of the 75 mg injections were pyrogenic. Average temperature increase in the 14 pyrogenic responses was 3.2° F (range 1.8 to 4.5° F); average lag time was 7 hours (range 4 to 11 hours); average temperature peak was reached at 11 hours (range 8 to 16 hours); and average time required for temperature to return to normal was 25 hours (range 20 to 48 hours).

Average time of intravenous infusion of steroid pyrogen in subjects developing fever from the 25 mg dose was 130 minutes; unsuccessful infusions averaged 90 minutes. In addition, three other subjects in whom intravenous injection of steroid pyrogen in this amount over short periods (30 minutes) induced little or no significant fever, developed high fevers when the infusion period was increased to 120 minutes or more. Figure 4 demonstrates an example of this type of response in one of these subjects during two different infusion periods. All subjects in whom intravenous administration of steroid failed to provoke fever later developed intense pyrogenic reactions when receiving equivalent or lesser amounts of steroid by intramuscular injection.

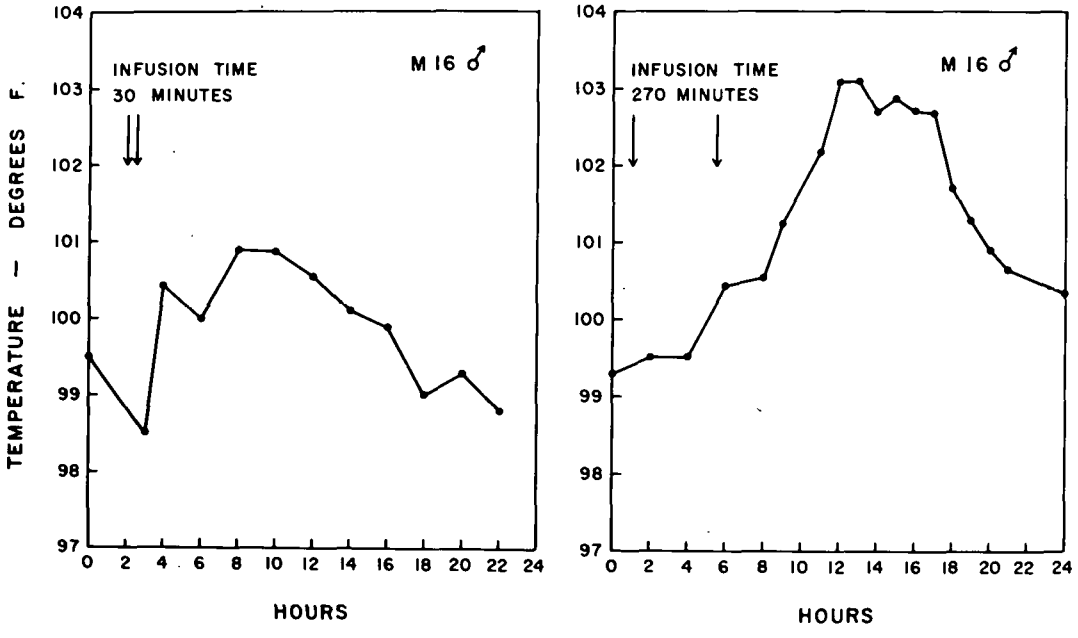

Figure 4. Example of the relation between duration of infusion of steroid pyrogen and febrile response observed in some subjects.

Intravenous injection of steroid pyrogen evoked overt inflammation along the course of the catheterized vein (arm) in many subjects whether or not fever developed. In some cases inflammation intensified or persisted for several days to a week or more after the day of injection; in 3 instances in which fever was induced, clear cut inflammation was not manifest although minimal degrees of venous irritation may have escaped detection. Control infusions of 1 to 3 ml of ethanol, if given slowly, were accompanied only by minimal or no signs of vein irritation; injection of small amounts of steroid in alcohol more rapidly than indicated could not be done because of considerable discomfort along the course of the vein.

Oral administration of steroid pyrogen. Nine subjects received a total of 10 doses of steroid by oral administration as follows: one of 25 mg; three of 50 mg; three of 100 mg; and three of 200 mg. No significant temperature elevation developed in any subject although all were known to develop pyrogenic reactions to intramuscular administration of 25 mg of steroid pyrogen. In two additional subjects with cirrhosis of the liver and proved esophageal varices, oral administration of 200 mg of steroid each failed to induce fever.

Leucocyte response to steroid pyrogen administration. The leucocyte response to steroid pyrogen administration was examined 23 times in 14 patients receiving 25 mg injections of the compound (21 intramuscular, 2 intravenous injections). During the 2 intravenous injections (infusion periods 120 minutes) leucocyte and differential counts were made at 30-minute intervals for the first 8 hours after injection, and subsequently at 4- to 12-hour intervals until the fever and leucocyte count had returned to normal. Following the intramuscular injections of steroid, counts were made at one-hour intervals for 12 hours and then at 2-hour intervals until the leucocytosis had subsided. In 5 of the subjects receiving intramuscular administration of steroid, leucocyte counts were also made at 5- to 10-minute intervals during the first hour after injection.

All subjects developed significant leucocytosis during the course of steroid fever. This leucocytosis was principally granulocytic in nature with marked shifts to the left, reached levels 2 to 4 times normal and usually subsided as fever regressed. Neutropenia was never observed at any time during the course of fever in these subjects.

Metabolism experiments. Observations on the calorigenic effect of steroid pyrogen injection were made in 5 subjects in whom fever was induced by intramuscular administration of 25 mg of 11-ketopregnanolone. In 3 subjects this relation was studied by means of indirect calorimetry (Benedict-Roth apparatus) with measurements of oxygen consumption being made at frequent intervals during control periods and during the 12 to 20 hours following steroid pyrogen injection. In 2 subjects calorimetric determinations were made alternately with the Benedict-Roth device and the Tissot closed system apparatus during the same frequent intervals.

Calorimetric measurements following steroid pyrogen injection revealed uniform sharp increases in heat production coincident with the development of fever. In 2 subjects these increases preceded the onset of perceptible chills; in the other 3 subjects, brief and slight to moderately vigorous episodes of chills were observed coincident with or slightly preceding the onset of the noted increases in oxygen consumption. Table 1 shows the temperature and metabolic responses of 5 subjects during control periods (-2, 0 hours) and at intervals during the succeeding 12 hours following steroid pyrogen injection.

Observations on sweating activity were made in 3 subjects during control periods and at 2-hour intervals for 10 to 14 hours after steroid injection, utilizing the iodine-starched paper test applied to the following areas: forehead, palms, soles, axillae, abdomen, forearm and inner thighs. Except for test periods, all 3 subjects rested quietly in bed, and were covered only with single sheets. Ambient temperatures averaged 75 to 80° F. During control periods sensible perspiration was not evident but could be evoked by local application of dry heat (infra-red lamp); within 4 hours after steroid administration local sweating responses to applied heat were not detectable but could be induced by intradermal injection of nicotine picrate indicating persistance of axon reflex sweating responses.[11] Spontaneous sudomotor activity was not manifest during the latent period but did resume at the time of temperature decline, as expected.

Table 1

RELATION BETWEEN TEMPERATURE AND METABOLIC RATE IN 5 SUBJECTS DURING THE 2 HOURS PRECEDING AND THE 12 HOURS FOLLOWING SINGLE I.M. INJECTION OF PYROGENIC AMOUNTS OF 11-KETOPREGNANOLONE

Subjects		Control hours		Hours after steroid injection						
		-2	0	1	2	4	6	8	10	12
1	Temp. F°	98.6	98.4	98.4	98.4	98.6	100.4	103.1	104.4	103.7
	Cal/M²/hr	36.6	35.5	35.5	36.4	37.2	42.6	48.4	50.8	47.9
2	Temp. F°	98.4	98.6	99.0	99.0	99.1	100.0	100.4	102.0	101.5
	Cal/M²/hr	37.6	35.9		34.8	35.9	39.8	41.4	44.2	44.7
3	Temp. F°	99.0	99.1		99.5	99.7	100.2	102.2	102.2	101.9
	Cal/M²/hr	36.9	36.4		35.3	35.3	40.6	42.1	43.9	43.2
4	Temp. F°	99.7	99.1	99.1	98.6	98.6	99.3	103.1	103.5	102.7
	Cal/M²/hr	32.8	35.2	35.8	36.6	37.2	37.5	51.0	46.6	44.9
5	Temp. F°	97.8	97.8	98.1	98.1	98.1	99.0	101.5	102.7	102.0
	Cal/M²/hr	31.1	31.6		31.6	30.4	31.7	40.6	42.4	39.3

Plasma transfer studies. Twelve attempts were made to demonstrate the presence of a transferable thermogenic substance, comparable to the leucocyte pyrogen[12-16] in plasma taken from subjects during the course of steroid-induced fever. Six of these experiments were conducted as follows: Fever was induced in the volunteer subject by intramuscular injection of 25 mg of 11-ketopregnanolone on 4 or 5 successive days. At the peak of the temperature response on each occasion (about 9 to 12 hours after injection) a 400 to 600 ml phlebotomy was done utilizing Fenwal ACD packs. The plasma was immediately separated off into Fenwal transfer packs and the red cell mass was then returned to the donor. Plasma was stored unfrozen for several days until amounts totaling 1000 ml or more were accumulated at which time the plasma pool was rapidly (30 minutes) reinfused into the afebrile original donor subject and temperature was recorded at 1/2 to 1-hour intervals for the next 24 hours. The temperature at the time of plasma withdrawal in these 6 subjects varied but averaged approximately 103° F. Four other plasma transfer experiments differed only in that plasma withdrawals were made at approximately the end of the latency period (5 to 7 hours) rather than at the peak of steroid fever. Average donor temperature at the time of phlebotomy in these 4 experiments was approximately 100° F. In all 10 experiments the period of time which elapsed between collection of the last plasma sample in each pool and the reinfusion of the plasma pool into the afebrile donor varied from 2 to 5 days. No significant temperature elevation developed in the 24 hours immediately following plasma reinfusion in these 10 subjects and none experienced any of the symptoms such as headache, malaise, myalgia and anorexia sometimes associated with the development of steroid fever.

In 2 additional subjects plasma in the amounts of 175 and 350 ml was withdrawn at the peak of steroid fever and immediately (within 45 minutes) infused into 2 afebrile recipients (patients with limited life expectancy due to malignancy but in good general condition) neither of whom developed significant temperature elevation in the succeeding 72 hours.

Steroid pyrogen induced inflammation. Observations on the histologic characteristics and time of occurrence of the local inflammation induced by this class of compounds, were made by biopsy examination of local sites of steroid injection in 10 subjects at intervals of 6 hours to 6 days after administration of 11-ketopregnanolone (5 subjects), etiocholanolone (3 subjects) or lithocholic acid (2 subjects). A total of 21 biopsies was made, most of which were obtained at 6, 12, 24 and 72 hours sequentially from subjects who received multiple injections of steroid. Control biopsies of sites of vehicle injection alone were also made at appropriate intervals.

Biopsies - 6 hours. (Four subcutaneous biopsies obtained from 4 subjects during the latent period of steroid fever).

One specimen was devoid of significant inflammation as were subsequent tissue samples obtained at 12 and 24 hours after injection of pyrogenic amounts of 11-ketopregnanolone. It is assumed that in this patient steroid injections were given more deeply than anticipated and that subcutaneous biopsies missed presumed areas of deeper inflammation. Three specimens showed acute severe inflammatory changes characterized by intense infiltration of polymorphonuclear leucocytes, predominantly neutrophils with variable amounts of eosinophils, throughout the dermis and subcutaneous layers, with marked edema and cellular infiltration of small blood vessels, capillaries and skin appendages. In the mid-dermis a number of arterioles had their lumens filled with neutrophils and a few histiocytes; in others, inflammatory cells gave the appearance of migrating through vessel walls into the surrounding subcutaneous tissue; in several small

muscular arteries at a deeper level, a severe angiitis was present. Foci of inflammatory cells were scattered through the fatty tissue.

Biopsies - 12 to 24 hours. (Ten biopsies obtained from 5 subjects during the peak of fever (approximately 12 hours) and after decline of temperature to normal (approximately 24 hours)).

In one subject both biopsies showed no inflammation, as noted above. The remaining 4 subjects showed inflammatory changes at 12 to 24 hours of the kind noted at 6 hours, though generally more severe. The cellular infiltrates were entirely polymorphonuclear in character; in some sections all dermal and subcutaneous layers were massively infiltrated with such neutrophils, with accompanying severe panniculites and angiitis. Edema was marked, vascular engorgement was pronounced and there was, in some areas, microabscess formation. In general, no distinction could be made between intensity of inflammation at 12 to 24 hours.

Biopsies - 36 to 72 hours. (Two biopsies from 2 subjects receiving injections of etiocholanolone and 11-ketopregnanolone; 2 biopsies from 2 subjects receiving injection of lithocholic acid).

In the patients injected with the neutral steroids the deep dermis and adjacent subcutaneous tissues showed persistent severe inflammation accompanied by necrosis and accumulation of fibrin, degenerating neutrophils and eosinophils, and necrotic fat and fibrous connective tissue. One section showing a severely inflamed area contained a large blood vessel filled with purulent and thrombotic material and showing extensive necrosis and degeneration of its walls. Other sections showed isolated foci of subacute and chronic inflammation around skin appendages and along the course of blood vessels; one sample showed muscle tissue and adjacent fat in which many of the small blood vessels were surrounded by clusters of lymphocytes; a few clusters of lymphocytes were also present between the muscle fibers. In general, inflammation was acute and severe with cellular infiltrates still largely polymorphonuclear in type.

In the patients injected with lithocholic acid the inflammation was altogether more severe with extensive destruction of skeletal muscle, fibrous tissue and fat; these tissues being replaced by necrotic material, hemorrhage and sterile abscess formation. Fat necrosis was marked and some medium sized vessels showed massive infiltration of acute inflammatory cells throughout the entire thickness of walls. Eosinophils were not prominent.

Biopsies - 4, 5, 6 days. (Three biopsies taken at 4, 5 and 6 days respectively from 1 subject).

Inflammatory changes in these sections were comparable in severity to those characterizing biopsy samples taken earlier.

Representative photomicrographs of inflammatory changes noted at local sites of steroid injection, after regression of fever, are shown in Figure 5.

COMMENT

Production of fever by steroid pyrogens follows in general a limited dose-response relationship exemplified in these studies by enhancement of the magnitude and consistency of thermogenic reactions to 11-ketopregnanolone over the five-fold dose range depicted in Figure 1. The minimum amount of this steroid (and related compounds) which produces significant fever with some regularity is about 10 mg. The thermogenic action of lesser doses, although occasionally pronounced, is in general variable while amounts approximately 25 mg or more usually provoke intense pyrogenic reactions. In this study 11-ketopregnanolone was, in appropriate doses, almost

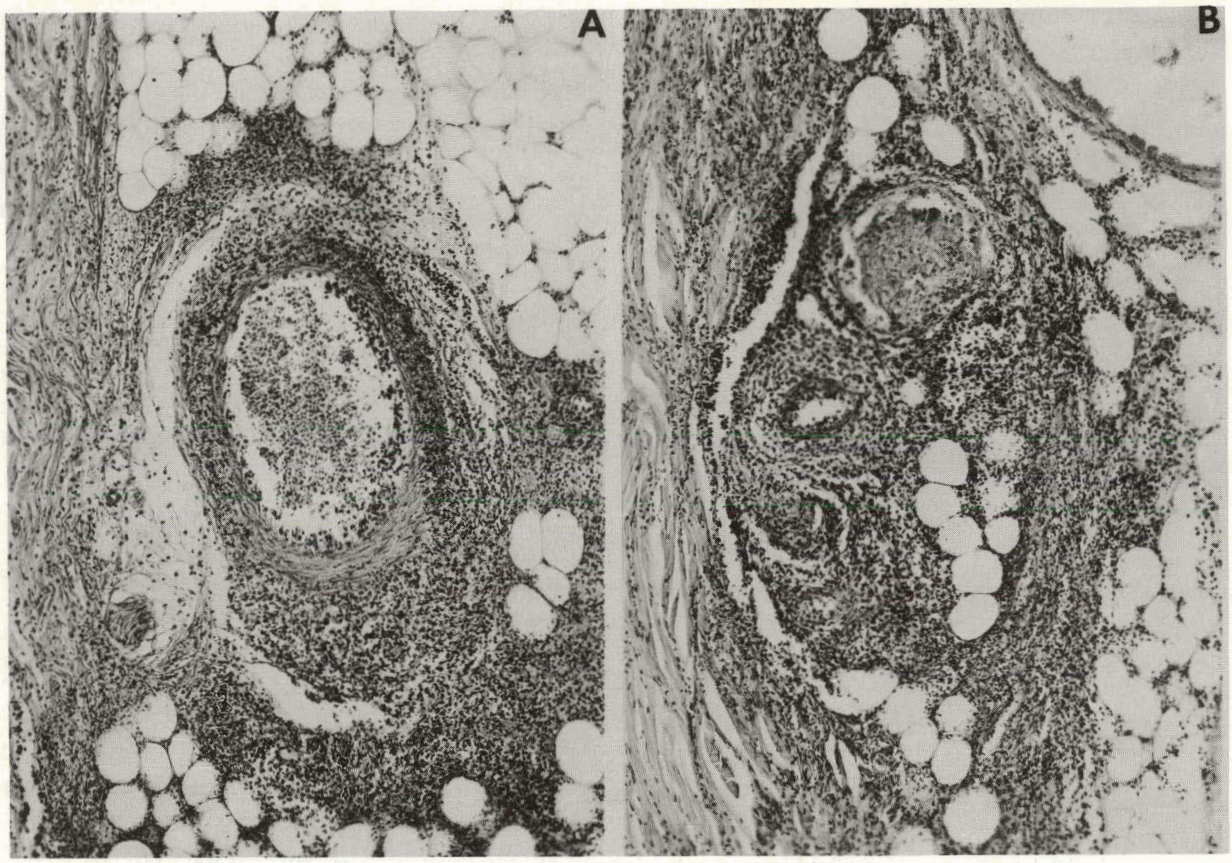

Figure 5. Photomicrographs of biopsies obtained at local sites of steroid pyrogen injection demonstrating intense local inflammatory changes, with massive polymorphonuclear leucocyte infiltrates, panniculites, myocitis, etc. The biopsy on the left (5A) was obtained at 24 hours; that on the right (5B) at 36 hours after steroid injection—i.e., after the febrile response had subsided.

always effective in producing fever when administered by intramuscular injection. Thermogenic responses to intravenously administered steroid, however, were less consistent even in those subjects known to respond with high fever to intramuscular injection of equal or lesser amounts of steroid. The reasons for these irregular responses are not clear, although in certain subjects they may be related in part to the duration of the intravenous infusion. Oral administration of large amounts of steroid pyrogen was ineffective in producing fever even in patients with significant liver disease and apparent portal-systemic shunting of blood. It is likely that such results reflect primarily the enormous capacity of the liver to conjugate or in other ways inactivate administered steroid. It may be recalled that very large doses of progestational hormones are required for demonstration of the thermogenic action of these compounds following oral, as compared with intramuscular, administration.[17]

A number of neutral and acidic steroids, especially of the 5β-H type, possess powerful cytotoxic properties. Although first recognized 150 years ago, in the context of studies on the hemolytic effects of bile, this cytotoxic action of steroids has been shown to extend to a wide variety of cell types including lymphocytes, leucocytes, spermatozoa, protozoa, bacteria, marine eggs, hepatocytes, and epithelium of the stomach, gallbladder and intestine, etc.[17] The intense

local inflammatory reactions elicited in this study by injections of 5β-H steroid pyrogens undoubtedly represent another expression of the cytotoxic activity. The lesions induced by etiocholanolone, 11-ketopregnanolone and lithocholic acid were shown on histological examination to be well advanced within 6 hours (and probably earlier) after administration of steroid and to be present for periods as long as 6 days. From the gross appearance of such reactions, severe focal inflammation may persist for even longer periods of time in many subjects. It is evident, therefore, that steroid pyrogen induced inflammation commences well before the onset of temperature elevation, and persists long after fever has subsided and most of the administered steroid has been absorbed, metabolized and excreted.[18] The role that these steroids play in the inflammatory process thus appears to be primarily one of chemical inciting agents, with further evolution of the local reaction presumably being dependent on other factors, including in part the severity of the initial stimulus.

The prolonged latent period of fever evoked by intramuscular or intravenous injection of steroid pyrogens contrasts sharply with the short lag time (15 to 90 minutes) of fever induced by bacterial endotoxins. This delay in endotoxin fever has been attributed to the time required for release of leucocyte pyrogen into the blood stream and its entry into the intrathecal space. It is this leucocyte material which probably represents the proximate stimulus for fever in endotoxin-treated hosts and which accounts for the immediate temperature elevation induced in afebrile recipients by plasma transfer from appropriately treated donors.[12,15] Failure in these and other studies[19] to demonstrate the presence of a comparable substance in the plasma of patients with steroid fever represents an important difference between the biological properties of bacterial and steroid pyrogens and one which may denote different modes of action of these fever-producing agents. Demonstration of endogenous pyrogen in man has proved to be difficult, however,[20] and alternative explanations for the failure to demonstrate this substance in plasma during the course of fever induced by steroids are possible. For example, still larger volumes of plasma may be necessary to demonstrate its presence in steroid fever; leucocyte containing plasma may be required for this demonstration; plasma taken at times different from those examined in this study may be necessary since endogenous pyrogen may appear only transiently in plasma during the course of steroid fever; different rates of infusion of appropriate donor plasma may be important for its demonstration; i.e., very slow infusion as compared with the rapid injections employed in this study (however, see[19]); the short periods of storage of plasma before transfusion, employed in this investigation, may inactivate leucocyte pyrogen in man (although this is not the case with leucocyte pyrogen derived from experimental animals), etc. The studies reported here shed no light on these various possibilities.

The overriding significance of central structures in thermo-regulation has been firmly established;[21] the recent studies of Benzinger have, in particular, clarified the relative importance of central and peripheral thermoreceptors in temperature homeostasis in man,[22,23,25] These investigations, utilizing the precise methods of gradient calorimetry and cranial thermometry, have shown that heat production (i.e., "chemical" heat regulation[23]) is a process in which both peripheral (skin) as well as central receptors operate; in warm environments, however, hypothalamic cells act as terminal receptor organs directly, without apparent stimuli from the skin. Heat dissipation (i.e., "physical" heat regulation[23]) is mediated through hypothalamic thermoreceptors possessing the unique capacity of translating minute temperature changes of blood into effector stimuli for appropriate vasomotor and sudomotor responses.

The physiologic events operative in steroid fever can be described in terms of these processes. The end of the latent period in these studies was marked by a significant increase in heat production, as measured by oxygen consumption (Table 1). The stimulus for this thermogenesis (due largely to shivering, although other factors may be operative[26]) was probably peripheral in origin. Although thermal conductance measurements were not made, it is reasonable to adduce this from the fact that the ambient temperature (below 80° in all cases) together with the expected cutaneous vasoconstriction would favor excitation of skin cold receptors[25] and the subsequent occurrence of overt chills, as observed. The increase in metabolic rate which developed with the onset of fever paralleled the rise in temperature and was consistent with it (approximately 7 per cent increase in metabolism/degree F temperature elevation), a relationship fitting the pattern of thermoregulation described as "continuous proportional control" by Hardy.[21] Since heat production of the magnitudes observed would not per se seriously disturb temperature homeostasis, concurrent suppression of heat dissipating mechanisms must have developed in order to account for the temperature elevations noted. Central stimulation of sudomotor activity with resultant heat loss, through evaporation, would be the major compensatory mechanism expected and its failure to operate, as noted, provided the essential background presumably for the development of fever in these subjects. It should be emphasized that suppression of sudomotor activity alone, without associated thermogenesis, could not account for fever, since active sweating is a process which is not necessary for heat dissipation under normal thermal loads—anhydrotic subjects, for example, experience little difficulty with fever unless exposed to external heat, or unless internal heat stresses (i.e., exercise) are invoked. Regression of steroid fever probably reflects direct hypothalamic drive of sudomotor and vasomotor heat dissipating mechanisms under the powerful stimulus of high internal temperature,[23,24] gradual diminution of central "set point" temperature[21,25] resulting from metabolic disposal of the substance acting on hypothalamic thermoregulatory structures, or both.

The nature of the proximate thermogenic stimulus in steroid fever remains speculative, and experimental study of this question appears severly hampered by the species specificity of the phenomenon.[7,26] Despite the intensity and rapid progression of steroid induced inflammatory reactions, certain observations recorded in this and earlier studies[1,2,4,18] make it improbable that their relation to the development of fever is a direct one—i.e., one in which the sole expression of the biological activity of 5β-H steroids is the production of local inflammation, with fever being a consequence entirely of the latter process. These observations include, among others, the frequency of venous inflammation without fever following use of the steroid Viadril;[17] the severe inflammatory responses unaccompanied by fever, evoked by taurolithocholic acid in man;[28] the temperature elevation induced by ovarian secretion of progesterone,[29,30] a cyclic physiologic occurrence not apparently attributable to inflammatory actions of this hormone;* the thermogenic effect of orally administered progestational hormones;[17] the disparity in time noted between regression of febrile and inflammatory responses; and the regularity with which repeated steroid pyrogen injections evoke fevers of the same general characteristics and magnitude, as in the tolerance studies (see above and [4]), together with the undoubted fact that such recur-

*It should be emphasized that progesterone has significant cytotoxic (hemolytic) activity in vitro, however,[17] and that local reactions do develop when it is administered intrumuscularly to man. Inflammatory effects in vivo are presumably precluded by direct secretion of the hormone into the blood stream.

rent fevers develop against a background of persistent local inflammation induced by preceding injections of steroid. These considerations make it unlikely that steroid induced inflammation per se accounts for fever; an indirect role, i.e., involving alterations in the rate of absorption of injected steroid, etc.,[6,35] for local inflammatory reactions in the thermogenic process cannot be excluded, however.

The possibility that the centrally acting thermogenic stimulus represents a specific product of steroid induced inflammation regularly formed at the site of injection, i.e., one derived from leucocytes, or formed through other specific steroid-tissue interactions at inflammatory sites, has been examined experimentally, within the limits possible in humans, as described above. Failure, in the plasma transfer experiments, to demonstrate such a substance, does not, as discussed above, preclude its formation during the course of steroid fever; it does however make it reasonable to focus attention at present on the possibility that injected steroid itself, or a derivative,[31] plays an active role in the production of fever. This role could involve direct effects on central structures controlling both heat production and heat loss mechanisms in man;* or effects on the latter processes alone with transient suppression of sudomotor and vasomotor heat dissipation providing the essential background against which any of a variety of stimuli for heat production would lead to fever.

A biological action of this type for certain steroids would represent a pharmacological counterpart of the antipyretic activity of cortisol and the lesser though significant temperature lowering actions of natural estrogens[17] and would be consistent with both the well known central nervous system actions of steroids in general and with other recognized drug effects on thermoregulatory structures,[34] as well. A mechanism of fever production considered along these lines moreover would provide a reasonable basis for considering the manner in which steroids such as progesterone and etiocholanolone participate in physiologic[29,30,35] and pathologic[36-38] temperature elevations in man.

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*The close anatomic relationship between hypothalamic structures controlling thermoregulation and TSH release[32] may be of special interest in this regard particularly in view of the influence of thyroid hormone on the ratio of 5α-H/5β-H derivatives produced during the metabolism of steroids.[33]

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ESTROGEN PHARMACOLOGY. I. THE INFLUENCE OF ESTRADIOL AND ESTRIOL ON HEPATIC DISPOSAL OF SULFOBROMOPHTHALEIN (BSP) IN MAN*

By

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This report‡ describes the influence of natural estrogens on liver function, with special reference to sulfobromophthalein (BSP) excretion, in man. Pharmacological amounts of the hormone estradiol consistently induced alterations in BSP disposal which were shown, through the techniques of Wheeler and associates[2,3] to result from profound depression of the hepatic secretory transport maximum (T_m) for the dye. Chromatographic analysis of plasma BSP components revealed increased amounts of BSP conjugates during estrogen as compared with control periods, implying a hormonal effect on cellular processes concerned with transport of dye from hepatocytes into biliary canaliculi. Estriol, an in vivo transformation product of estradiol, also impaired hepatic disposal of BSP in man. According to unpublished data of Hertz[4§] esterone, ethinyl estradiol and equine estrogens act similarly and it is likely that other C-18 steroids of both physiologic and synthetic origin have this property as well. These observations define a new biological action of natural estrogens in man, further substantiate the role of the liver as a site of action of these hormones[5] and probably account, in part, for the impairment of BSP disposal which characterizes pregnancy[6] and the neonatal period.[7-10]

METHODS

Steroid solutions were prepared by dissolving crystalline estradiol and estriol in a solvent vehicle containing 10 per cent N,NDMA (N,N-Dimethylacetamide)[¶] in propylene glocol. Estradiol was soluble in a concentration of 100 mg per ml; estriol, in a concentration of 20 mg per ml. These, together with appropriate control solutions, were sterilized by filtration at room temperature and administered to patients by intramuscular injection. All subjects were housed on a metabolic ward during the study and a number were maintained on fixed diets as required by concurrent investigations. The principal clinical diagnoses were rheumatoid arthritis and related connective tissue disorders. The observations reported here were made during a series of studies designed initially to examine the potential use of pharmacological amounts of natural estrogens as therapeutic or immunosuppressive[11-13] agents in man; the periods of estrogen administration therefore varied considerably from subject to subject. Liver function was examined

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‡These data were presented at the 77th Annual Meeting of the Association of American Physicians, May 6, 1964, and a preliminary report published in the Transactions of that society.[1]

§We have recently been privileged to review earlier unpublished observations of Dr. Roy Hertz, National Cancer Institute, National Institutes of Health, on BSP metabolism in cancer patients undergoing intensive estrogen therapy. These observations demonstrate rapid and consistent abnormalities in BSP disposal, as described in this report.

¶Eastman Organic Chemicals, Division of Eastman Kodak Co, Rochester, New York.

in these patients by means of the following tests: per cent esterification of cholesterol, direct and indirect bilirubin, cephalin and thymol flocculations, thymol turbidity, prothrombin time, serum albumin and globulin, retention of BSP in plasma 45 minutes after intravenous administration of 5 mg per kg body weight, and the following serum enzymes: alkaline phosphatase, lactic dehydrogenase (LDH), glutamic pyruvic transaminase (SGPT) and glutamic oxaloacetic transaminase (SGOT). BSP infusion studies, chromatography of plasma BSP components and liver biopsies were performed in several subjects as described below.

Thirty-one patients were treated with either estradiol or estriol. Two were males; 29 were females, most of whom were postmenopausal. Estradiol was administered to 25 patients (24 females) in amounts of 5 to 200 mg per day for 3 to 41 days. Estriol was administered to 6 patients (5 female) in amounts of 7 to 40 mg per day for 6 to 10 days. Injections of control vehicle solutions were given to each of 13 patients in amounts of 2 ml per day for 30 days.

RESULTS

Liver function tests were performed twice in control periods in all subjects and at frequent intervals during control injections and estrogen treatment.

Control vehicle-treated subjects. No alterations in any liver function test were detected in the 13 control patients either during the 4-week treatment period or in the succeeding 3 months. N,NDMA does produce some hepatotoxicity in experimental animals when given in doses greatly exceeding those employed in this study;[14,15] however, this substance is approved for use clinically as a solubilizing agent for antibiotics in a concentration of 50 per cent and no hepatotoxicity has, to our knowledge, been reported. The studies of Hertz[4] which were done with aqueous or other estrogen preparations in any case obviate the possibility that BSP impairment reported here is related to solvent vehicle action.

Estrogen-treated subjects. The 31 patients in this group were selected on the basis of normal hepatic function with particular reference to BSP retention (6 per cent or less in 45 min), alkaline phosphatase (6 Bodansky units or less), bilirubin and appropriate serum enzymes during control periods.

The following tests remained unchanged from control values in all subjects during estrogen treatment and in follow-up periods ranging from 3 to 12 months; cephalin and thymol flocculations, thymol turbidity, per cent esterified cholesterol, prothrombin time, direct and indirect bilirubin, LDH, SGOT and SGPT. Total serum proteins showed changes consistent with estrogen-induced plasma volume increases, but albumin to globulin ratios were unaltered from control values. Serum alkaline phosphatase rose to abnormal levels (in excess of 6 Bodansky units) in nearly 50 per cent of estrogen treated patients and remained elevated until injections ended. In this study BSP retention in plasma exceeding 6 per cent 45 minutes after injection of the standard dose (5 mg per kg body weight) was considered abnormal. By this criterion, 24 of 25 subjects receiving estradiol and 4 of 6 receiving estriol showed impaired BSP disposal during steroid treatment. This impairment developed within the first week of treatment, reaching levels as high as 30 per cent retention. When studied daily in some subjects, BSP retention was evident within 24 hours of steroid treatment. According to Hertz[4] massive intravenous infusions induced abnormal BSP disposal within hours of treatment. Impairment in BSP excretion tended to remain constant at about the level reached in the first week of estrogen administration; in some subjects the per cent BSP retention by the standard test diminished somewhat with continued hormone

treatment although it never reverted to control values until steroid was discontinued. Abnormal retention of dye invariably receded when estrogen treatment ended.

The steroid treatment schedule, per cent BSP retention and level of serum alkaline phosphatase in the control period and at the end of the period of estrogen administration in each subject are shown in Table 1. These data, which are graphically represented in Figure 1, demonstrate that pronounced impairment in BSP disposal frequently occurred in the absence of significant alteration in serum alkaline phosphatase; elevated enzyme levels however were not observed unless there was concurrent abnormal BSP retention. Figures 2 and 3 show specific examples of the impairment in BSP disposal induced by small amounts of estradiol and estriol in 4 subjects.

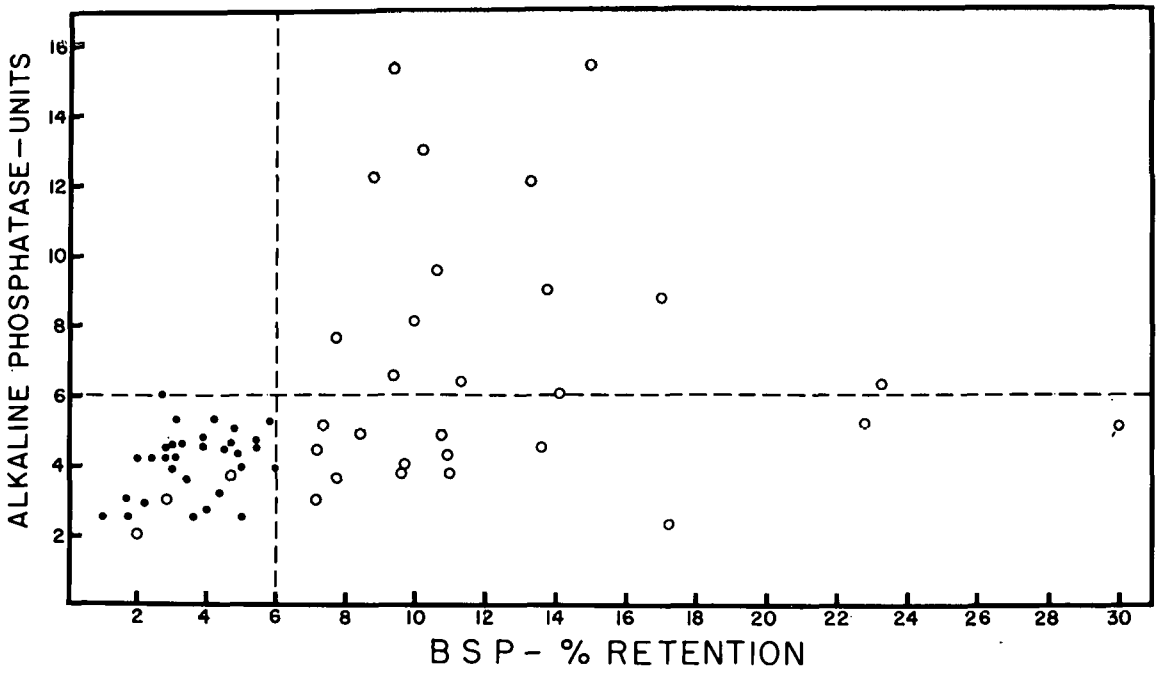

Figure 1. Serum levels of alkaline phosphatase plotted against per cent retention of BSP during control and estrogen periods in 31 subjects. Control values are indicated by solid dots; estrogen period values by open circles. Normal values for both indices are enclosed in the lower left quadrant.

In order to define this estrogen effect in more detail, the methods of Wheeler and associates[2,3] were employed to determine the hepatic secretory transport maximum (T_m) and hepatic relative storage space (S) for BSP during control and estradiol periods in 6 subjects. These methods and their application to studies of BSP metabolism have been described in detail[2,3] and will not be reviewed here. The values for hepatic secretory transport maximum (T_m) and hepatic relative storage space (S) for BSP during control and estradiol periods in each patient are recorded in Table 2.

BSP components in plasma obtained during comparable (i.e., plasma sampled at the same time in the same portion of the injection sequence) infusion periods were examined chromatographically[10,16,17] in 4 subjects as follows: 2 ml of plasma were extracted with approximately 10 ml acetone; the extract was dried in vacuo; the residue was redissolved in 0.5 ml distilled

Table 1

TREATMENT SCHEDULE, BSP TESTS AND SERUM LEVELS OF ALKALINE PHOSPHATASE DURING CONTROL AND ESTROGEN PERIODS IN 31 SUBJECTS

Patient	Treatment		BSP retention %		Alkaline phosphatase Bodansky units	
			Control	Estrogen	Control	Estrogen
LG	Estriol	7 mg/day x 8	1.7	7.2	3.0	3.0
JZ	Estriol	10 mg/day x 7	2.2	4.7	2.9	4.7
OH	Estriol	10 mg/day x 9	3.9	7.4	4.5	5.2
JW	Estriol	20 mg/day x 6	1.7	2.0	2.5	2.0
SO	Estriol	20 mg/day x 8	3.0	8.4	4.6	4.9
MH	Estriol	40 mg/day x 10	4.7	9.4	4.6	6.6
JR	Estradiol	5 mg/day x 6	1.0	2.8	2.5	3.0
RB	Estradiol	5 mg/day x 6	2.4	7.7	4.2	7.7
BM	Estradiol	5 mg/day x 7	2.7	10.6	6.0	9.6
EC	Estradiol	10 mg/day x 18	3.3	7.2	4.6	4.4
OH	Estradiol	25 mg/day x 5	4.4	9.7	3.2	4.0
ST	Estradiol	25 mg/day x 9	4.8	10.8	5.0	4.8
PD	Estradiol	25 mg/day x 12	4.9	11.0	4.3	4.3
CT	Estradiol	50 mg/day x 5	3.9	9.4	4.8	15.3
DH	Estradiol	50 mg/day x 17	3.1	11.0	4.2	3.8
LT	Estradiol	100 mg/day x 3	4.2	15.0	5.3	15.4
CM	Estradiol	100 mg/day x 7	2.8	10.2	4.2	13.0
EK	Estradiol	100 mg/day x 11	3.6	17.2	2.5	2.3
VS	Estradiol	100 mg/day x 11	3.4	13.3	3.6	12.1
BP	Estradiol	150 mg/day x 5	2.0	14.1	4.2	6.0
MS	Estradiol	200 mg/day x 3	5.4	13.6	5.0	4.5
EB	Estradiol	200 mg/day x 22	6.0	23.3	3.9	6.3
AT	Estradiol	200 mg/day x 23	5.0	22.9	3.9	5.2
AS	Estradiol	200 mg/day x 23	4.5	7.8	4.4	3.6
WC	Estradiol	200 mg/day x 24	5.4	13.7	4.7	9.0
GS	Estradiol	200 mg/day x 27	2.8	9.6	4.5	3.8
JK	Estradiol	200 mg/day x 30	3.0	10.0	3.9	8.1
HE	Estradiol	200 mg/day x 31	5.0	30.0	2.5	5.1
LM	Estradiol	200 mg/day x 33	4.0	8.8	2.7	12.2
ML	Estradiol	200 mg/day x 34	5.8	11.3	5.2	6.4
EL	Estradiol	200 mg/day x 41	3.1	17.0	5.3	8.8

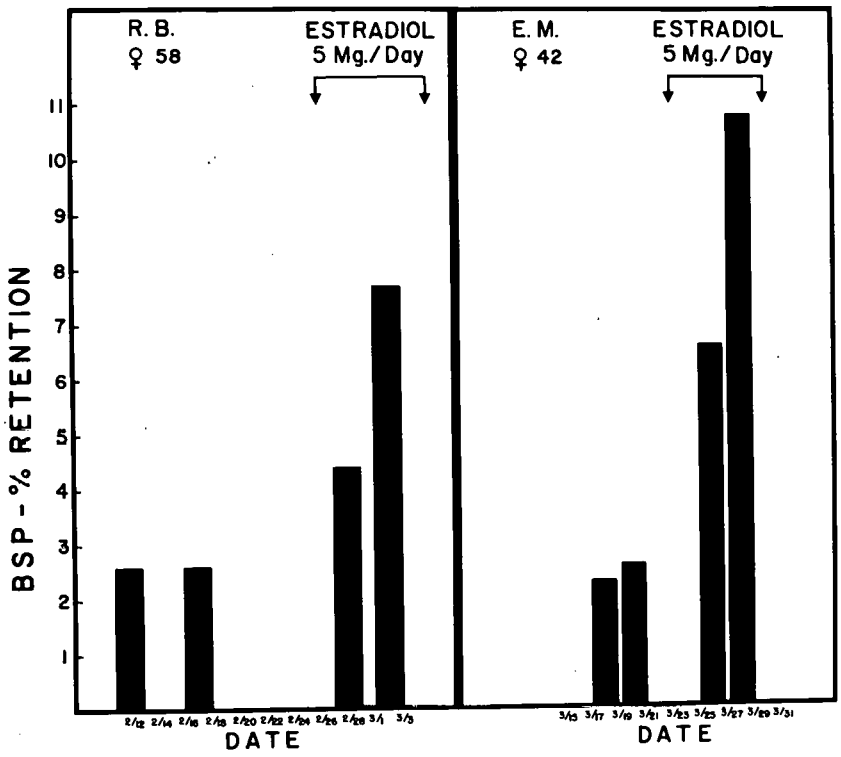

Figure 2. Effects of estradiol on BSP retention in 2 subjects receiving 5 mg per day for 6 days (R.B. - tests on days 2 and 5) and 7 days (E.M. - tests done on days 2 and 4). Pre-treatment BSP tests are shown on the left in each graph.

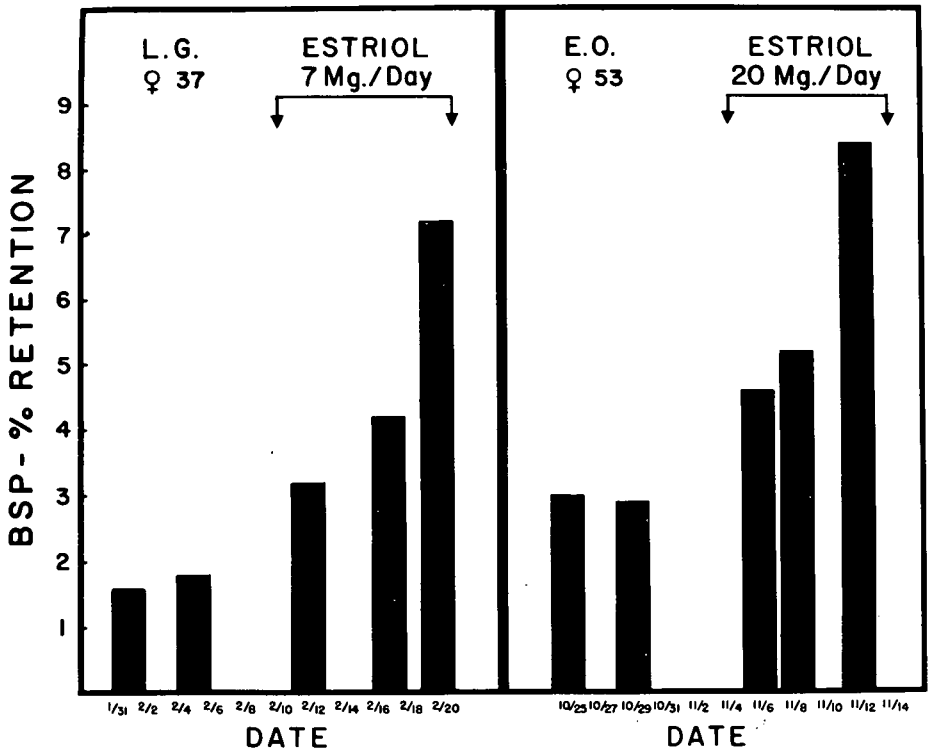

Figure 3. Effects of estriol on BSP retention in 2 subjects receiving 7 mg per day for 8 days (L.G. - tests on days 2, 6 and 8) and 20 mg per day for 8 days. (E.O. - tests on days 2, 4 and 8). Pre-treatment BSP tests are shown on the left in each graph.

Table 2

PLASMA RETENTION (45'), HEPATIC SECRETORY TRANSPORT MAXIMUM (T_m) AND HEPATIC RELATIVE STORAGE SPACE (S) FOR BSP IN 6 PATIENTS DURING CONTROL AND ESTROGEN TREATMENT PERIODS

Patient - Treatment	Plasma retention at 45 minutes %		Hepatic secretory transport maximum (Tm) mg/min		Hepatic relative storage capacity (S) mg/mg %	
	Control	Estrogen	Control	Estrogen	Control	Estrogen
CT - Estradiol 50 mg/day for 5 days	3.9	9.4	8.70	2.57	131.5	108.0
BP - Estradiol 150 mg/day for 5 days	2.0	14.1	7.60	2.55	54.6	40.5
DH - Estradiol 50 mg/day for 17 days	3.1	11.0	6.15	2.70	58.5	117.5
CM - Estradiol 100 mg/day for 7 days	2.8	10.2	6.50	0.34	37.8	77.6
EK - Estradiol 100 mg/day for 11 days	3.6	17.2	6.60	4.05	54.2	68.0
VS - Estradiol 100 mg/day for 11 days	3.4	13.3	6.40	2.55	27.5	58.7

water, applied to a 5 cm strip on Whatman no. 1 paper and chromatographed for approximately 16 hours in a descending system containing glacial acetic acid, water and N-propanol (1:5:10). Following chromatography, BSP compounds on the chromatogram were identified by exposure to ammonia fumes; appropriate areas were marked and eluted; and the BSP compounds quantitated at 580 mμ in alkaline buffer in a Beckman DU spectrophotometer. The proportions of free and conjugated BSP were determined by the relation of the individual to the combined optical densities and are reported for 4 subjects to the nearest whole figure in Table 3. The assumption was made that the chromatographic losses and optical densities of free and conjugated BSP were

Table 3

CHROMATOGRAPHIC ANALYSIS OF BSP COMPOUNDS IN PLASMA

Patient	Control period		Estrogen period	
	Free %	Conjugated %	Free %	Conjugated %
CT	94	6	80	20
BP	93	7	50	50
DH	85	15	57	43
CM	85	15	40	60

equal.[17] The developed chromatograms from control and estradiol periods in one of the 4 subjects are shown in Figure 4. The 3 trailing BSP bands shown were ninhydrin positive. Three conjugates were not detected in all 4 subjects but the number observed during control and estrogen periods in each case was always equal.

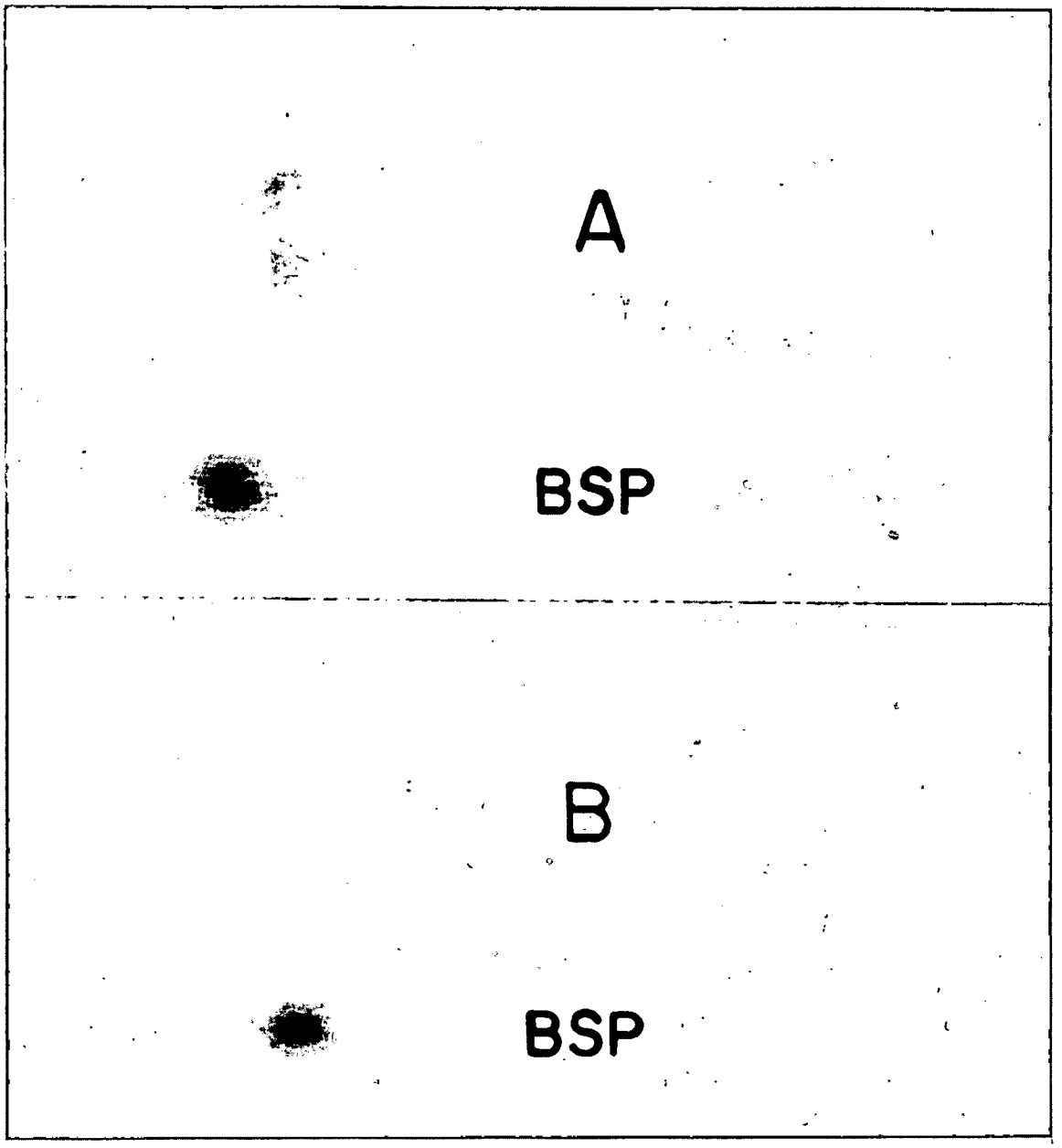

Figure 4. Developed (ammonia vapor) chromatograms of BSP compounds in plasma during comparable BSP infusions before (A) and after (B) estrogen treatment in one patient. Origins are at the right; standard BSP spots are labeled. In the control period (A), a heavy free BSP and 3 trailing lighter BSP bands can be seen; the 3 trailing bands were ninhydrin positive. In the estrogen period (B) all 4 BSP components are also evident.

Percutaneous biopsies of the liver were obtained during control periods and at the end of 17 to 39 days of treatment with 50 to 200 mg per day of estradiol in 6 patients undergoing intensive experimental therapy with this hormone. Tissue samples were fixed in formalin, stained with hematoxylin and eosin, for iron and for fibrous tissue (Mallory) and examined by light microscopy. Liver biopsy was normal in 2 subjects in both control and estrogen periods. The remaining subjects had a variety of nonspecific or slight changes during control periods, consisting of mild hemosiderosis, slight "reactive" and fatty changes, minimal proliferation of Kupffer cells, etc. No significant alteration in these hepatic findings was induced by the estrogen treatment described; specifically, bile casts were not seen and biliary canaliculi remained inconspicuous. Electron microscopic examination of biopsy samples was not available at the time of these studies.

DISCUSSION

This study demonstrates that the natural estrogen, estradiol, impairs hepatic excretory capacity for the dye sulfobromophthalein (BSP) in man. Following administration of this hormone abnormal BSP retention, as determined by the standard single injection test, developed rapidly and persisted as long as hormone injections continued. Hepatic disposal of BSP reverted to normal when estradiol treatment ended; the rate of recovery of hepatic excretory capacity for this dye is not known with certainty although it is probably relatively rapid. This may be adduced from the fact that BSP tests became normal in all subjects 1 to 4 weeks after estrogen treatment was stopped despite the administration of large amounts of steroid in an injection vehicle from which delayed absorption could be anticipated. The only other index of hepatic function significantly altered by estrogens in this study was the serum alkaline phosphatase, which was increased to abnormal levels in approximately 50 per cent of steroid treated subjects. Other serum enzyme activities indicative of direct hepatocellular damage remained unaltered, and BSP retention, as noted, developed in many subjects in whom no change in serum alkaline phosphatase occurred. Estriol, an in vivo transformation product of estradiol, also impaired hepatic excretory capacity for BSP in man. No attempt was made to compare the relative potency of estradiol and estriol with respect to this action. It is evident from these studies and those of Hertz (vide supra) that this biological property probably characterizes a variety of C-18 steroids of physiologic and synthetic origin.

Determination of the hepatic secretory transport maximum (T_m) and hepatic relative storage space (S) for BSP according to the methods of Wheeler and associates coupled with chromatographic analysis of the amounts of free and conjugated dye in plasma provides a quantitative description of the hepatic processes cencerned with BSP metabolism.[2,3,6,10,16-22] By inference, such data also provide information on the probable site of action of substances affecting disposition of this dye. Hepatic relative storage space for BSP did not alter in a consistent fashion during estrogen administration; there was, nevertheless, a tendency for the value of (S) to increase and this was occasionally pronounced. Hepatic secretory transport maximum for this dye, however, was markedly depressed in all subjects following hormone treatment; in 1 subject hepatic T_m decreased nearly 95 per cent reaching a level in the estradiol period within the range observed in patients with congenital disorders of hepatic excretory function such as the Dubin-Johnson syndrome.[3] Chromatographic analysis of BSP compounds in plasma revealed uniformly higher levels of conjugated BSP during estrogen administration as compared with

control periods, thus discounting major hormonal impairment of conjugation processes for this dye or of transfer of dye from sinusoid to hepatocyte (although these data do not entirely exclude some estrogen action on these processes). It is reasonable to infer therefore that the alteration in hepatic excretory capacity for BSP which is induced by estrogens is attributable largely to defective transport of BSP compounds across hepatocyte membrane bordering canalicular spaces where excretion takes place. It is relevant to note that the hepatic T_m calculated by these techniques is a composite figure which represents an average of the transport maxima for the several BSP components which have been identified;[3] it is possible that estrogens impair hepatic transport of each of these compounds to a different degree. Failure of estradiol and estriol to induce hyperbilirubinemia in these studies is of interest and may be attributable to the magnitude of the excretory T_m for this substance;[23] it does not imply that capacity of the liver for disposal of this pigment remains intact during such treatment, since defects in hepatic disposal of bilirubin may be demonstrable only with appropriate infusion studies as Soffer has shown in pregnant women[24] (and as Combes and associates[6] have shown for BSP). Similarly the absence, by light microscopy, of overt alterations in hepatic parenchyma during intensive estrogen treatment does not preclude demonstration, with appropriate histochemical and electron microscopic techniques, of the hepatocellular changes described by others in patients and experimental animals treated with synthetic steroids which induce similar defects in hepatic function.[25-28]

These data define a new biological property of natural estrogens and indicate that the liver may be, especially in pregnancy, an important site of action of these hormones in man. Together with the observations of Hertz (vide supra) they also demonstrate that impairment of hepatic excretory function is a steroid hormone action which is not restricted solely to synthetic compounds of unnatural configuration such as methyl testosterone, 17α-ethyl-19-nortestosterone, 9-fluoro-11β-hydroxymethyl testosterone and related substances.[29-33]

The amounts of estradiol administered to most patients in this investigation considerably exceeded those secreted in the gravid state (although not all amounts (Figure 1) exceeded the total daily output of estrogenic steroids in pregnancy);[34-36] however, no attempt was made to determine either the minimal dose of estradiol required to impair BSP excretion or the influence of chronic administration of physiologic doses of hormone on this index of liver function in men. Moreover, the doses of estriol used in this study were in fact well within the amounts of this specific metabolite produced during the last trimester of normal human pregnancy;[34,36] consideration of the potential clinical relevance of these observations is therefore warranted.

Although standard texts still suggest that pregnancy does not alter hepatic function in humans, elevation of serum levels of alkaline phosphatase are common during the last trimester;[37-40] delayed disappearance of infused bilirubin during this period has been demonstrated;[24] standard BSP tests may be abnormal at this time;[41,42] and, as Combes and associates[6] have shown recently, the hepatic secretory transport maximum for BSP is significantly lower in gravid as compared with normal women. Hepatic T_m for the dye rapidly reverts to normal in the postpartum period and these authors have suggested that impaired hepatic disposition of BSP is mediated by the increased hormonal levels of pregnancy.[6] The results of the present study are consistent with this view and indicate that estrogens in particular may account wholly, or in part, for the maternal hepatic functional alterations described. The possibility that progesterone may modify this estrogen action in pregnancy merits examination; in preliminary studies from

this laboratory pharmacological amounts of this C-21 steroid alone, have failed to alter the hepatic T_m for BSP in humans.

It has also been known for some time that premature as well as normal infants have impaired excretory capacity for BSP in the neonatal period.[7-9] Oppe and Gillis[9] have shown that the most marked abnormality, in plasma disappearance curves of BSP in the newborn, occurs in the second or excretory phase (as in the Dubin-Johnson syndrome)[43] and that recovery of hepatic capacity for disposal of the dye occurs rapidly, regardless of the gestational age or birth weight of the infant. BSP-glutathione conjugating enzyme activity in fetal and neonatal rats and in maternal rats at term is low according to the studies of Combes and Stakelum[10] who have suggested that delayed BSP clearance in neonatal children may be due in part to inadequate development of this conjugating system. From other studies in newborn and older children Vest[44] concluded that this delay was caused primarily by impairment of the secretory mechanism for the dye. The human fetus is exposed to extremely high levels (approximately 10 times maternal blood levels)[45] of estrogens, particularly estriol[34,36] near term. This fact, in light of the data reported here, suggests that impaired excretory capacity for BSP in the neonatal period may represent, in part, a residual effect of the intense hepatic exposure to these hormones in utero.

The relation of this estrogen action to disorders of liver function apart from the maternal-fetal excretory impairments noted is speculative, since it is not known whether this effect in man is self-limited, leads to morphological changes or predisposes the liver to injury from otherwise innocuous processes. It may prove fruitful however to consider the possibility that endogenous hormones of this type or their transformation products[12] might have some relation to the apparent severity of hepatitis in pregnancy[46-48] or the high female incidence of post-hepatitis cirrhosis[49-52] and hepatotoxic drug reactions.[53]

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ESTROGEN PHARMACOLOGY. II. SUPPRESSION OF EXPERIMENTAL IMMUNE POLYARTHRITIS*

By

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(With the technical assistance of Evelyn Damgaard and Elizabeth Laves)

A generalized inflammatory joint disease develops in rats after intradermal injection of complete adjuvant. The studies of Pearson, Waksman and associates[1,2] suggest that this disease is immunological in nature having features of a delayed hypersensitivity type of reaction. The recent demonstration[3] that passive transfer of the arthritis can be effected by living lymphoid cells from sensitized donors strongly supports this view.

Natural estrogens significantly alter the morphology and function of hemopoietic and lymphoid tissues and may influence the occurrence and intensity of certain immunological reactions.[4-6] In the present study it is shown that these steroid hormones significantly diminish the incidence of adjuvant-induced arthritis in rats.

PROCEDURES AND RESULTS

Sprague-Dawley rats 12 weeks of age or older were used throughout. Weights of males were approximately 300 to 400 grams at the time of inoculation; weights of females were approximately 200 to 300 grams. Equal numbers of both sexes were used in each experimental group. Adjuvant was prepared by dispersing heat-killed Mycobacterium Butyricum (DIFCO) in light mineral oil (Bayol 55, ENCO) and administered as a single intradermal injection of 0.1 ml at the base of the tail. Steroids were dissolved in 0.5 per cent N,H dimethylacetamide (EASTMAN ORGANIC CHEMICALS) in propylene glycol. Steroid and appropriate solvent vehicle control solutions were sterilized by cold filtration and injected subcutaneously in the axillary fold in the amounts indicated in the table. Animals were examined for arthritis on alternate days after adjuvant inoculation for 30 to 60 days. A separate control group treated with appropriate amounts of solvent vehicle alone was used in each experiment. The χ^2-test was utilized to evaluate the significance of the data.[7]

Table 1 summarizes all experiments, indicating the mode of treatment, number of animals observed, the number of per cent of animals developing arthritis with an evaluation of the significance (p-values) of the difference between incidence of arthritis in the steroid treated groups as compared with the control group in each experiment.

DISCUSSION

These experiments demonstrate that the natural hormone estradiol consistently diminishes the incidence of inflammatory joint disease evoked by adjuvant injection in rats. This protective effect of estradiol was apparent over a wide dose range and in a variety of treatment schedules (Experiments I-IV); moreover, estimation of the severity of the arthritis on the basis of number

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of inflamed joints and duration of involvement indicated that estradiol treated animals had much milder disease, as compared with controls. Estrone and estriol, in vivo transformation products of estradiol, were as effective in suppressing arthritis as the parent hormone. Since the transformation, estradiol ⟶ estriol, is not reversible in vivo, the protective effect of the latter steroid suggests, as in the hepatic action of these hormones,[8] that other natural or synthetic C-18 steroids may have this property as well. Though not studied in detail, it appears likely on the basis of these (Experiment VI) and earlier observations,[4] that the extent of estrogen protection depends in part on the intensity (i.e., amount of Mycobacterium incorporated in the inoculum) of the antigenic challenge. The minimum amount of estrogen required for protection is not known with certainty, although in view of the ameliorative effect of gestation on this immunologic disease,[9] it is probably well within the range produced during normal rat pregnancy.

The incidence of arthritis evoked in rats treated with progesterone, testosterone and cortisol in the schedule and amounts shown in Experiment V was not significantly different from that in the control group. It should be noted that in this experiment, these hormones were administered prior to inoculation only, in order to compare their effects with those of the natural estrogens. According to the studies of Pearson and Wood,[1] cortisol does diminish the severity of adjuvant arthritis when administered after inoculation.

The mechanism by which estradiol, estrone and estriol confer protection in adjuvant arthritis is not known. Since lymphocytes appear to be involved in the pathogenesis of this disease,[2,3] the well-known depleting effects of estrogens on lymphoid tissues such as the thymus[10,11] may account in part for the protective action of these hormones. Another possible mechanism for this estrogen action is suggested by these experiments. It has been shown[12] that excision of the adjuvant injection site and its draining lymph nodes shortly after inoculation does not prevent subsequent development of arthritis, indicating that the immunologic reaction leading to arthritis is quickly initiated. The fact (Experiments III and V) that estradiol diminishes the incidence of arthritis when administered in the latent period either starting on the day of inoculation, or 5 and 10 days thereafter suggests a hormonal effect directly on the capacity of involved tissues to react to the inflammatory stimulus in this disorder. Such an action would be consistent with earlier observations of Lurie,[13] showing that both pregnancy and estrogens transiently suppress the intensity of delayed skin reactions in known tuberculin positive animals.

Estrogen administration and/or pregnancy has in other studies been shown to lessen the incidence and severity of experimental allergic thyroiditis, diminish the intensity and frequency of delayed skin reactions to thyroglobulin and tubercle protein and prolong the survival and function of trophoblast implants and of skin grafts in experimental animals and man.[4,13-16] The present study with an inflammatory joint disease considered to be immunological in nature represents another experimental context in which estrogens are shown to suppress the manifestations of an apparent delayed type immune reaction, and which suggests that such hormones may mediate the related effects of pregnancy on such phenomena. The possibility that this estrogen action has clinical relevance may be considered in view of the extraordinary increase in estrogen production (principally estriol) which characterizes human pregnancy[17] and the profound ameliorative effect of gestation on both human[18] and experimental[9] polyarthritis.

Table 1

TREATMENT SCHEDULES AND INCIDENCE OF ADJUVANT ARTHRITIS IN RATS RECEIVING VARIOUS STEROIDS

Exp.	M. Butyricum in adjuvant dose/animal	Group	Treatment	No. treated	No. with arthritis	% arthritis	p value
I							
A	0.4 mg	Control	—	47	33	70.2	
B	0.4 mg	Estradiol	0.5 mg TIW 3 weeks pre- + post-inoculation	49	17	34.7	< 0.005
II							
A	0.6 mg	Control	—	40	39	97.5	
B	0.6 mg	Estradiol	1.0 mg q.d. 10 days pre- + TIW 4 weeks post-inoculation	40	19	47.5	< 0.005
C	0.6 mg	Estradiol	1.0 mg q.d. 10 days pre- + TIW 1 week post-inoculation	24	9	37.5	
D	0.6 mg	Estradiol	1.0 mg q.d. 10 days up to day of inoculation	32	15	46.9	
III							
A	1.0 mg	Control	—	20	12	60.0	
B	1.0 mg	Estradiol	0.6 mg TIW 2 weeks bgn. 5 days post-inoculation	13	3	23.1	< 0.025
C	1.0 mg	Estradiol	0.6 mg TIW 2 weeks bgn. 10 days post-inoculation	12	2	16.7	

Table 1 (continued)

Exp.	M. Butyricum in adjuvant dose/animal	Group	Treatment	No. treated	No. with arthritis	% arthritis	p value
IV							
A	0.6 mg	Control	—	20	16	80.0	
B	0.6 mg	Estradiol	0.1 mg TIW 2 weeks pre-inoculation	20	8	40.0	<0.01
C	0.6 mg	Estradiol	0.3 mg TIW 2 weeks pre-inoculation	22	7	31.8	
D	0.6 mg	Estradiol	0.6 mg TIW 2 weeks pre-inoculation	21	8	38.1	
E	0.6 mg	Estradiol	1.0 mg TIW 2 weeks pre-inoculation	22	7	31.8	
V							
A	0.6 mg	Control	—	22	18	81.8	
B	0.6 mg	Estradiol	1.0 mg TIW 3 weeks pre-inoculation	22	10	45.4	<0.005 (estrogens only)
C	0.6 mg	Estrone	1.0 mg TIW 3 weeks pre-inoculation	22	6	27.3	
D	0.6 mg	Estriol	1.0 mg TIW 3 weeks pre-inoculation	22	7	31.8	
E	0.6 mg	Cortisol	1.0 mg TIW 3 weeks pre-inoculation	22	15	68.2	
F	0.6 mg	Progesterone	1.0 mg TIW 3 weeks pre-inoculation	43	31	71.8	
G	0.6 mg	Testosterone	4.0 mg TIW 3 weeks pre-inoculation	22	13	59.1	
H	0.6 mg	Estradiol	1.0 mg TIW 4 weeks from day of inoculation	22	8	36.4	
VI							
A	0.2 mg	Control	—	30	9	30.0	
B	0.2 mg	Estrone	1.0 mg q.d. 10 days pre-inoculation	30	—	0.0	<0.005

The following abbreviations are used: TIW for 3 times per week; q.d. for daily.

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